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Research paper

Comparative in vitro biocompatibility testing of polycyanoacrylates and poly(D,L-lactide-co-glycolide) using different mouse fibroblast (L929) biocompatibility test models

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Abstract

Comparative in vitro cytotoxicity studies of polyalkylcyanoacrylates (PCA) and poly(D,L-lactide-co-glycolide) (PLGA) were performed. Four PCAs of different alkyl chain lengths and two commercially available PLGAs were assayed in several cell culture models recommended by the International Standard Organization (ISO). Different polymer preparations, two extraction methods evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and LDH (lactate dehydrogenase) release, two indirect contact methods, for example the agar overlay, and a direct contact method were used. Test procedures included light microscopy, the vital stain neutral red, the MTT assay, based on the cleavage of a yellow tetrazolium salt (MTT) to purple formazan crystals by mitochondrial active cells, LDH release and scanning electron microscopy. All polycyanoacrylates tested showed cytotoxic effects with methylcyanoacrylate being the most toxic compound. Cytotoxic effects and proliferation inhibition decreased with increasing alkyl chain length of the PCAs. PLGA was very well tolerated by the cultured cells in all assays performed. The extraction method evaluated by the MTT assay seems to be the most appropriate method, as it showed a clear graduation of toxicity and allowed an estimation of the 50% inhibitory concentration (IC_{50}) values for all polymers tested.

Keywords: Direct contact method; Extraction method; In vitro cytotoxicity; L929 cells; Polyalkylcyanoacrylate; Poly(D,L-lactide-co-glycolide)

1. Introduction

For the last 20 years, drug delivery systems based on biodegradable polymers have been an important area of research [1] allowing the development of parenteral depot systems. These systems offer controlled release properties, avoiding the surgical removal of the spent devices [2]. The biomaterials need to be biocompatible and safe, which has to be demonstrated in extensive studies under in vitro and in vivo conditions. Biocompatibility testing is a very complex issue involving local tissue response, systemic toxic effects, blood compatibility, immunologic and carcinogenic reactions [3]. For drug delivery systems especially, local toxicity is an important concern. To reduce the extent of safety studies in laboratory animals, in vitro cell culture models have gained more importance recently.

Biocompatibility has been defined as "the ability of a material to perform with an appropriate host response in a specific application" [4]. For a specific biomedical application, for example a prosthesis, an ideal polymer would be stable within its physiological environment.

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Since polymers used for drug delivery systems are intentionally degradable, residual monomers, catalysts and additives as well as the degradation products play an important role in defining their biocompatibility [5]. For evaluation of systemic toxicity and mechanical damage caused by a biomaterial, safety studies in animals cannot be avoided, whereas cytotoxic properties of the polymers and their degradation products can be characterized by in vitro cell culture models.

To study the potential of these in vitro biocompatibility testing models, we have investigated the in vitro cytotoxicity of several biodegradable polymers, which were proposed for parenteral drug delivery systems. Poly(D,L-lactide-co-glycolide) (PLGA) is used in a wide range of biomedical applications, for example as suture material [6] or artificial skin [7], and is already licensed for parenteral drug delivery formulations [8]. A major advantage of PLGA is the well documented safety and tissue compatibility [9]. Polyalkylcyanoacrylates (PCA) are known to be bacteriostatic and hemostatic [10,11]. They have been used as tissue adhesives in surgery, for bone repair and as dental material for many years. Their application as drug carriers is a matter of debate due to potential adverse tissue reactions and cytotoxic effects [12–15]. Colloidal carriers based on PCA may, however, show different characteristics, since they can undergo phagocytosis. This aspect was not a subject of this investigation.

The degradation products of PLGA, monomers and oligomers of lactic acid and glycolic acid, are known to be well tolerated by various tissues [16] and are finally eliminated from the body through the Krebs cycle [17]. For PCAs, different degradation pathways have been described. Some authors postulate a degradation mechanism following a Retro-Knoevenagel reaction leading to formaldehyde and cyanoacetate [18,19]. Other groups found only a minor contribution of this formaldehyde-producing pathway [20]. Lenaerts et al. [21] suggested a degradation process where the polymeric backbone chain remains intact, but becomes increasingly hydrophilic by cleavage of the pendant ester groups, leading to a water-soluble product. This reaction was observed under physiological conditions where the PCA was degraded mainly by an enzymatic process.

Several procedures have been described involving cell culture techniques for preliminary biocompatibility evaluation of materials intended for medical application [22]. To harmonize in vitro cytotoxicity test methods, an ISO norm was created by the International Standard Organization [23]. This document classifies the in vitro cytotoxicity test methods into three categories based on the preparation of the test material as follows: (1) test of extracts prepared from the polymer; (2) indirect contact methods, where cells and the test material are separated by a protective layer, for example an agar layer; (3) direct contact methods where the cells are put directly in contact with the test material. We used these different in vitro biocompatibility testing methods suggested by the ISO norm, by preparing extracts from the polymers according to USP XXIII, by using the agar overlay method [24,25] and by seeding the cells directly onto polymer films. Mouse connective tissue fibroblasts L929 (ATCC cell line CCL1, NCTC clone 929) are a standard model for biocomatibility testing, since they are easy to cultivate and because of their favorable doubling time of about 24 h. L929 cells are widely used for cytotoxicity studies and are recommended by many standard institutions [23,25–27]. The viability of the cells was quantitated by a colorimetric assay for cellular growth, the MTT assay [28], based on the cleavage of a yellow tetrazolium salt (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by mitochondrial active cells. Moreover, we determined lactate dehydrogenase (LDH) release as an indicator of cell membrane damage to investigate if surface active degradation products are released. Morphological examination of the cells was carried out using phase contrast microscopy and scanning electron microscopy (SEM).

We investigated the cytotoxicity of PCA and PLGA, not by a chemical quantification of the degradation products but by their biological effect on cultured cells. The aim of our study was the evaluation of the in vitro cytotoxicity of PCA in comparison with PLGA using different cell culture methods recommended by the ISO norm.

2. Materials and methods

2.1. Polymers

Poly(D,L-lactide-co-glycolide) (50:50) RG 503 (molecular weight (M_w) = 35 000) and RG 505 (M_w = 69 000) were obtained from Boehringer Ingelheim (Ingelheim, Germany), while cyanoacrylate monomers were gifts from Sichel-Werke (Hannover, Germany). We used cyanoacrylates with different alkyl chain lengths, namely methyl- (MCA), ethyl- (ECA), *n*-butyl- (BCA) and *n*-hexylcyanoacrylate (HCA). As positive control, tin-stabilized PVC, recommended by the British Standard Institution [27], was employed.

2.2. Cells

A mouse connective tissue fibroblast cell line, L929 (DSM, Braunschweig, Germany) was cultured in Dulbecco's minimum Eagle medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (Gibco, Germany) and 2 mM glutamine. No antibiotics were added to the cell culture medium. The cultures were cultivated in an incubator at 37°C, 95%

r.h. and 10% CO_2 until cell monolayers attained confluence which occurred after 7 days. Thereafter, the cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Stock cultures were seeded in 60 mm diameter cell culture petri dishes (Nunc, Wiesbadan, Germany) at a density of 0.1×10^6 cells/petri dish and subcultured once a week. Assays were always performed in the exponential growth phase of the cells. The cell line was periodically assayed for mycoplasma using DAPI staining (4′,6-diamidino-2-phenylindole; Boehringer Mannheim, Mannheim, Germany) [29].

2.3. Extraction method

Extracts of the polymers were prepared according to United States Pharmacopeia USP XXIII [25] using 0.1 g polymer per ml serum-supplemented culture medium (DMEM) containing 2 mM glutamine. Random polymers RG 503 and RG 505 were milled in a freezer mill (Spex Freezer Mill, Spex Industries, Germany). The polycyanoacrylates (PCA) were synthesized in situ by adding the monomers to the extraction medium under stirring, resulting in the formation of aggregates.

The extracts were maintained in an incubator for 24 h at 37°C according to USP [25]. If necessary, the extracts were neutralized to exclude the effect of pH and filtered through a 0.2 μm membrane filter (Schleicher and Schuell, Dassel, Germany). The cells were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany) at a density of 2×10^3 cells/well, and after 24 h the culture medium (100 μl) was replaced with serial dilutions of the extracts. After an incubation period of 4 days, the viability of the cells was evaluated by the MTT assay [28].

2.4. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Deisenhofen, Germany) was dissolved in PBS (phosphate-buffered solution) at 5 mg/ml and filtered for sterilization. A total of 20 μl of this stock solution was added to each well, reaching a final concentration of 0.5 mg MTT per ml and the plates were incubated for 4 h. Unreacted dye was removed by aspiration and the purple formazan product was dissolved in 200 μl/well dimethyl sulfoxide (DMSO) and quantitated spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA) Reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at wavelengths of 570 and 690 nm. The spectrophotometer was calibrated using culture medium without cells. The relative cell growth (%) related to control wells containing cell culture medium without extracts was calculated by the following equation (A = absorbance):

$$\text{Relative cell growth (\%)} = \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100$$

2.5. Lactate dehydrogenase release

Polymer extracts were prepared by using 0.2 g polymer per ml extraction medium (PBS). The polymers were autoclaved at 121°C and 2 bar for 2 h. In preliminary experiments, we found that this relatively stressful extraction procedure was more effective in degrading the polymer samples than extraction temperatures of 70°C (data not shown). L929 cells were seeded at a density of 0.1×10^6 cells per well in 24-well culture dishes (Nunc, Germany). After 3 days, 0.2 ml of the extracts were added to confluent cell layers and were incubated for 1, 3 and 5 h with the cells. The LDH content of the samples was measured using a test kit (DG 1340-K, Sigma, Deisenhofen, Germany) and spectrophotometric determination (UV-160, Shimadzu, Kyoto, Japan). Control experiments were performed using 0.1% Triton-X 100 (ICN, Eschwege, Germany), which is known to completely lyse cell membranes.

2.6. Agar overlay method

The agar overlay assay was carried out according to USP [25] with slight modifications. L929 cells were seeded in 60-mm diameter cell culture plates at a density of 9×10^5 cells. After preincubation of 2 days, monolayers reached 80% confluence. Culture medium was replaced with 5 ml serum-supplemented culture medium containing 1% agar. We prepared extracts using 0.1 g polymer per ml of extraction medium by heating them for 24 h at 37°C [25] in an incubator containing 10% CO_2. Saline, serum-supplemented cell culture medium and sesame oil were used as extraction media. Filter paper (AP 2501000 Prefilter, Millipore, Eschborn, Germany) 1 cm in diameter was soaked with these extracts and placed on the solidified agar. After an incubation period of 24 h, the cultures were examined under an inverted light microscope, magnification × 100 (Nikon TMS, Nikon, Düsseldorf, Germany), using the cytochemical stain neutral red (Sigma). Vital cells take up neutral red whereas dead cells remain unstained. The biocompatibility of the test samples was evaluated using the rating scaling according to USP [25] ranging from 0 (no detectable reaction) to 4 (severe destruction of the cells), as outlined in Table 1. The zone index was determined by measuring the area of unstained cells surrounding the specimen. Lysis index was obtained by measuring the area of lysed or rounded cells under or beyond the specimen by light microscopy.

Table 1
Reactivity grades for agar overlay test

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Zone limited to area under specimen
2	Mild	Zone extends less than 0.5 cm beyond specimen
3	Moderate	Zone extends 0.5–1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond specimen

2.7. Indirect contact method

In the indirect contact method, 25–30 mg of the polymers were compressed to polymer tablets of 5 mm in diameter and 0.8–1.0 mm thickness using a laboratory press (Schwabenthan, Germany). PVC sheets were punched out in the same dimensions. The L929 mouse fibroblasts were seeded in 24-well culture dishes (Nunc, Wiesbaden, Germany) at a density of 5×10^4 cells/well. After 24 h, stainless steel inserts (Fig. 1) containing the polymer tablets were added. Cyanoacrylate monomers (25 mg) were dropped directly into the insert. The inserts protect the cells from direct mechanical damage, while the perforation ensures permanent contact between the polymer and the cell culture medium (1.5 ml/well). After an incubation period of 4 days, the viability of the cells was evaluated using the MTT assay described above. The formazan product was measured spectrophotometrically at a wavelength of 570 nm (UV-160, Shimadzu, Kyoto, Japan).

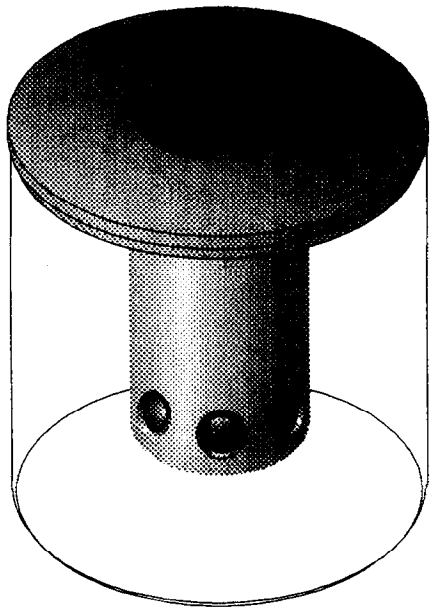

Fig. 1. Schematic illustration of a stainless steel insert (1.2 cm diameter) manufactured for a 24-well cell culture dish.

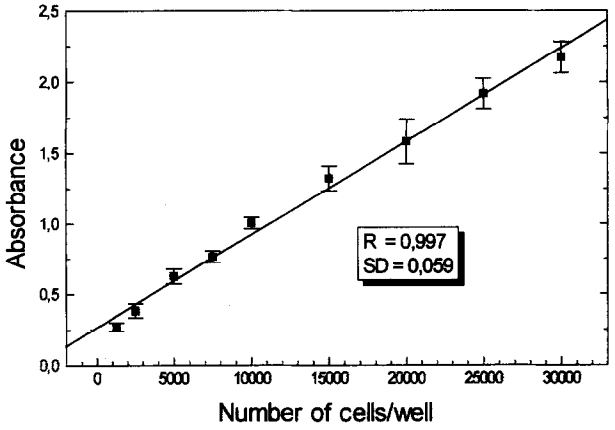

Fig. 2. Correlation of L929 mouse fibroblasts cell number with MTT cell function test ($n = 9$, results are given as mean values, bars represent standard deviation).

2.8. Direct contact method

PLGA films for the SEM studies were prepared by casting a 5% (m/v) dichloromethane solution on teflon-coated plates. The cyanoacrylate films were prepared by dropping the monomers onto glass coverslips. All polymer films were dried under vacuum for at least 72 h until a constant weight was obtained. Cells were seeded at a density of 3×10^5 cells/cm on the films and were incubated for 48 h. The cells were fixed in formaldehyde and buffered osmium–tetroxide solution 1%, and subsequently dehydrated in ethanol. The films were coated with gold (Sputter coater S 150, Edwards, Marburg, Germany) and examined using a Hitachi 501 scanning electron microscope (Hitachi, Tokyo, Japan).

3. Results

3.1. Extraction method

In preliminary experiments we tested the relationship between cell number, determined by cell counting (Hemocytometer, Neubauer) and the amount of MTT formazan generated. The results in Fig. 2 show that the adsorbance is directly proportional to the number of cells in the range from 1×10^3 to 3×10^4 cells/well.

After an exposure time of 4 days, all extracts prepared from PCA showed pronounced effects on the viability of the L929 fibroblasts similar to the toxic reference material PVC (Fig. 3), whereas the PLGA (RG 503, RG 505) extracts hardly influenced the cell viability. Evaluating the extract dilutions, a cytotoxicity graduation between the different PCAs is shown (Fig. 3) with MCA being the most toxic compound. The extraction method allows an evaluation of the concentration of the polymers which inhibits the cell growth by 50%. The inhibition concentration at which 50% of

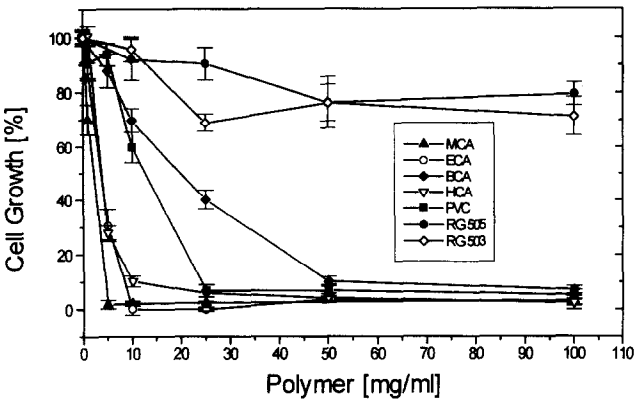

Fig. 3. Inhibition of L929 cell growth by polymer extracts and extract dilutions after an incubation time of 4 days ($n = 8$, results are given as mean values, bars represent standard deviation). Extracts were prepared with serum-supplemented cell culture medium maintained in an incubator for 24 h at 37°C.

all cells show toxic effects is usually referred as IC_{50}. MCA, ECA and HCA influenced the cell viability even more than the toxic reference material PVC, resulting in an IC_{50} of between 1 and 5 mg/ml. The IC_{50} of PVC was about 12 mg/ml extraction medium. BCA showed the lowest reduction of cell viability achieved by PCA, yielding an IC_{50} of about 20 mg/ml. The IC_{50} of PLGA could not be determined by this method, since the highest polymer concentration of 100 mg/ml showed cell viabilities of 70–80%.

3.2. Lactate dehydrogenase release

Fig. 4 shows LDH release of L929 cells by the influence of polymer extracts. Triton-X solution (0.1%), which is known to damage cell membranes, caused high LDH release set as 100%. With the exception of PVC and MCA extracts incubated for 5 h with the cells and ECA extracts incubated for 1 h with the cells, no significant increased LDH release was evaluated (t-test, $\alpha = 0.05$). MCA extracts incubated for 5 h obtained a slight increase in LDH release of 25% compared to the control. PVC extracts incubated for 5 h with the cells caused the highest amount of LDH release of 38%.

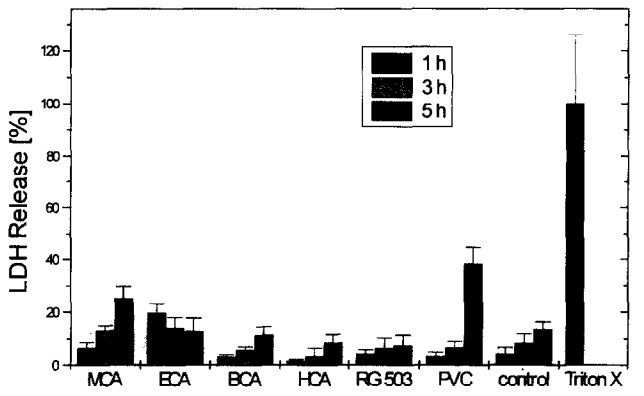

Fig. 4. LDH release of L929 cells after incubation with polymer extracts for 1, 3 and 5 h ($n = 3$, results are given as mean values, bars represent standard deviation). Extracts were prepared with PBS, heated to 121°C for 2 h.

3.3. Agar overlay method

The results of the agar overlay method are shown in Table 2. The zone index was determined using the cytochemical stain neutral red measuring the zone of decolorization around the sample. By examination of the monolayers under a phase contrast microscope, we measured the zone of lysed cells (lysis index). The PLGA copolymers did not influence the cell viability, while polycyanoacrylates affected the cell viability drastically, with MCA being the most toxic compound. MCA extracts always caused significant cytotoxicity, regardless of the extraction medium used. Evaluating the toxicity of extracts prepared with cell culture medium, MCA and HCA showed high scores of 4, ECA produced a moderate reaction and BCA showed no reaction. Determination of the extracts prepared with physiological saline resulted in severe cytotoxicity for MCA, slight reaction for ECA and BCA, and a moderate reaction for HCA. With the exception of HCA, the extracts prepared with sesame oil influenced the cell viability for each polymer significantly. The reaction to ECA appeared to be moderate to severe, while BCA and HCA caused moderate scores.

3.4. Indirect contact method

Preliminary experiments showed no influence of L929 cell growth by empty stainless steal inserts. The PLGA copolymers did not influence cell viability using the indirect contact method. All PCAs prevented cell growth to the same extent as the cytotoxic control PVC (Fig. 5), indicating the release of a soluble toxic compound. No toxicity range could be determined for the PCAs, since all PCAs tested decreased the cell viability to less than 10%.

3.5. Direct contact method

The adhesion and spreading of the L929 cells on PLGA films was not impaired compared to glass coverslips. Fig. 6 shows a scanning electron micrograph of a continuous layer of spreading fibroblasts on RG 503 films after 48 h. In contrast to the PLGA polymers, the adhesion of fibroblasts on the polycyanoacrylates was almost negligible, as demonstrated by their rounded shape and the low number of adhering cells (Fig. 6a–c). Without exception, the films prepared from PCA were characterized by a lack of attachment and spreading of the L929 fibroblasts.

Table 2
Observations of the agar overlay method after 24 h incubation of extracts prepared with cell culture medium, sodium chloride solution or sesame oil as extraction medium ($n = 3$)

	Cell culture medium		Sodium chloride		Sesame oil	
	Zone index	Lysis index	Zone index	Lysis index	Zone index	Lysis index
RG 503	0	0	0–1	0	0	0
RG 505	0	0	0	0	0	0–1
PVC	0	0	1	1–2	4	4
MCA	4	4	4	3–4	4	4
ECA	3	3	0 1	1	3–4	4
BCA	0–1	0	1	0–1	3	3
HCA	4	4	3–4	3	3	2

Extracts were prepared by incubation of the polymers with the extraction medium for 24 h at 37°C.

4. Discussion

4.1. Extraction method

To simulate the degradation process of different biodegradable polymers, we analyzed extracts, whereby leachable toxic components and degradation products of the material are dissolved in the extraction medium. An extraction method according to USP XXIII (0.1 g polymer per ml extraction medium) at 37°C for 24 h was used. The incubation time of 4 days ensures a contact of the extracts with the cells over several cell cycles in their exponential growth phase. We used the MTT assay to assess the cell viability since it was described to be a suitable method for the detection of biomaterials cytotoxicity [30,31]. The MTT assay is widely used due to its precision and reproducibility in measuring cell viability. The results in Fig. 1 show that the amount of formazan produced is directly proportional to the number of cells. These results correlate very well with radioisotope assays, e.g. [^{3}H]thymidine incorporation [28], and allow a rapid screening of a large amount of samples. The results obtained by the extraction method indicate that PLGA does not affect the viability and proliferation of L929 fibroblasts, confirming the well documented biocompatibility of this material [32]. All PCAs influence the fibroblasts' viability significantly. Some of them decrease the viability of the fibroblast even more than the positive control (PVC), resulting in an IC_{50} between 1 and 5 mg/ml for MCA, ECA and HCA, whereas the IC_{50} of PVC is about 12 mg/ml (Fig. 3). These results confirm the results of others, who found after a relatively short incubation time of 1 h, an IC_{50} of 0.4 mg/ml for BCA nanoparticle suspension and an IC50 of 1 mg/ml for HCA nanoparticles in a hepatocyte cell culture model [19]. Since no positive or negative controls were used, such as PLGA or PVC, their conclusion that both materials are of low cytotoxicity remains controversial.

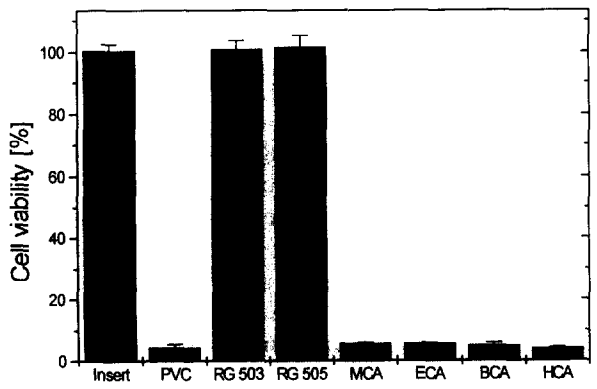

Fig. 5. Indirect contact test method. Effect of polymer tablets (25 mg) in stainless steel inserts on L929 cell layers ($n = 3$, results are given as mean values, bars represent standard deviation).

Our results correlate very well with in vivo studies of Woodward et al. [15] showing a marked histotoxicity for MCA and a decreased local toxicity with increasing alkyl chain length. Lehmann and coworkers [33], who investigated the nephrotoxicity of PCAs in dogs, also found MCA to be the most toxic homolog. Our extraction studies of PCA show the highest toxicity for MCA and the lowest toxicity for BCA. HCA was as toxic as ECA probably caused by the lower purity (90.1%) of this compound. The cytotoxic effect of the PCAs is not caused by SO_2 stabilizer, as we obtained the same toxic results when we washed the polymer with cell culture medium before the incubation with the extraction medium for 24 h (data not shown). Thus, we cannot confirm the results of Neupert and Welker [34] who proposed a correspondence of PCA toxicity with the amount of stabilizer. Tseng et al. [35] found that residual monomers of PCA are not responsible for their cytotoxicity. Our results are in accordance with the investigations of Leonard et al. [18] and Lehmann et al. [33] who suggested that the toxicity of PCAs may be caused by the degradation products. The extraction method used in this study allows a ranking of cytotoxic effects based on serial dilutions. The polymer concentration at which a cytotoxic effect is observed (IC_{50})

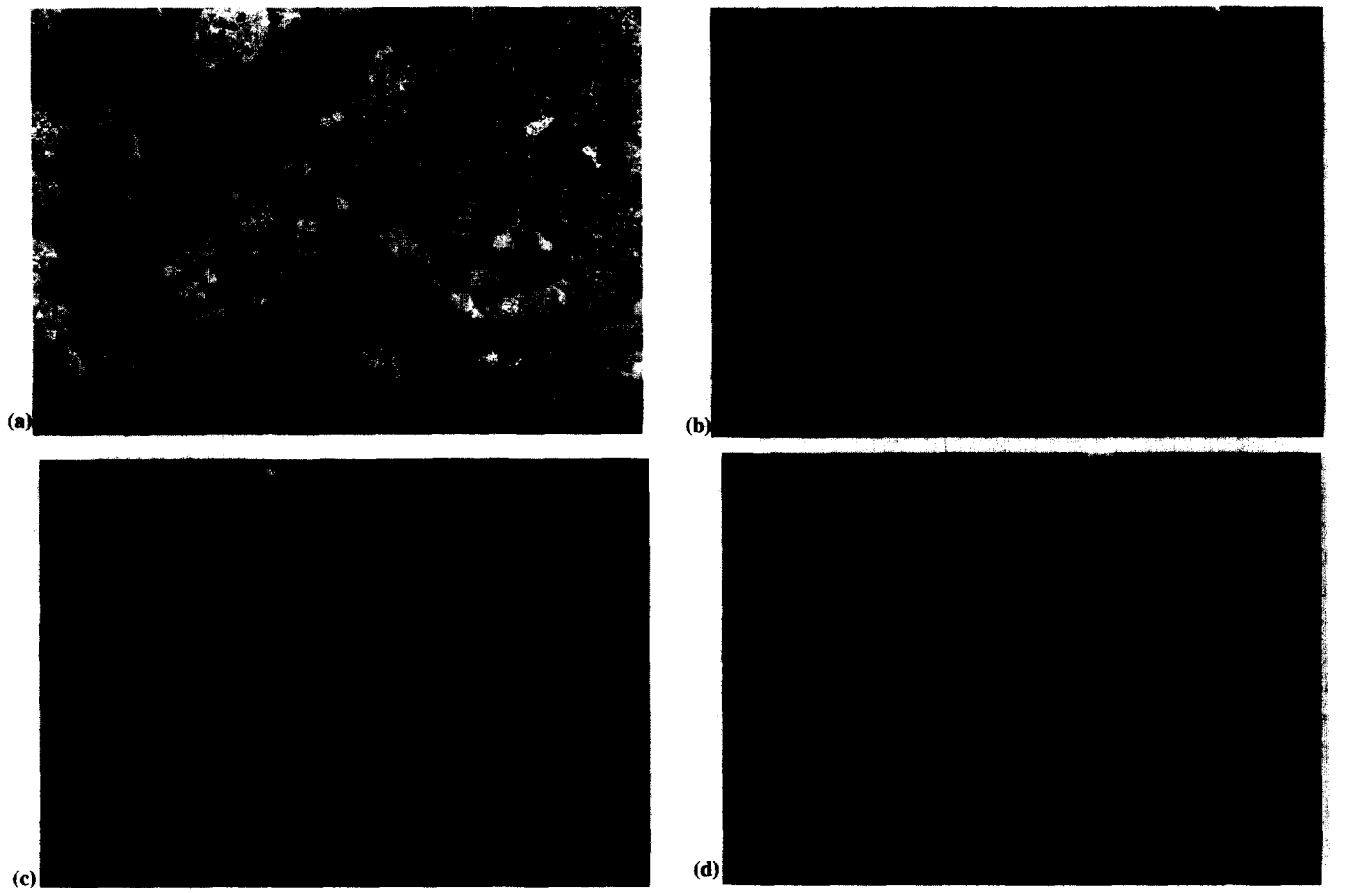

Fig. 6. (a) SEM picture of L929 cells on PLGA film after 48 h; (b) SEM picture of L929 cells on MCA film after 48 h; (c) SEM picture of L929 cells on ECA film after 48 h; (d) SEM picture of L929 cells on BCA film after 48 h.

gives the following rank order: PLGA > BCA > PVC > HCA > ECA > MCA.

4.2. Lactate dehydrogenase release

In preliminary experiments, we studied extracts prepared according to USP [25] using 1 mg/ml extraction medium (24 h, 37°C), but no increase in LDH release from L929 fibroblasts in any of the samples could be observed. Using a higher concentration of 0.2 g polymer per ml at 121°C for 2 h, the amount of LDH released from the L929 cells after incubation was negligible, suggesting that no surface active compounds are generated during polymer degradation. These findings are at variance with Couvreur [36] who observed cellular damage and LHD release of different cells caused by PCA nanoparticles. The nanoparticles were prepared by adding cyanoacrylic monomers to an aqueous solution containing dextran 70 (1%), glucose (5%) and 10^{-3} M HCl. Additives or the nanoparticles themselves possibly caused damage of the cell membranes.

4.3. Agar overlay method

In the agar overlay method, a solid agar layer protects the fibroblasts from mechanical damage. Leachable components from the polymer or degradation products diffuse through the agar to the cell monolayer. Using different extraction media, such as cell culture medium (DMEM supplemented with 10% FCS), physiological saline and sesame oil, we tried to differentiate cytotoxic effects between hydrophilic and lipophilic leaching compounds or degradation products on the cell monolayers [26]. MCA showed severe effects for all extraction media used. ECA yielded severe effects with sesame oil extracts, moderate reactivity for cell culture medium and slight effects for sodium chloride solution. For BCA extracts, we observed no reaction to slight effects for cell culture medium and sodium chloride solution but moderate reactions for sesame oil. HCA showed severe destruction of the cells for cell culture medium, moderate to severe effects for sodium chloride solution and mild to moderate effects for sesame oil. In

summary, the results of the agar overlay method confirm that the extraction method obtains a toxicity decrease with increasing alkyl chain length, with the exception of HCA, which shows higher toxicity than ECA and BCA, probably because of the lower purity of this compound.

The agar overlay method was questioned, since the penetration of chemical components might be delayed by the agar layer [37]. Our results demonstrate the importance of examining different extraction media since some polymers seem to contain lipophilic leachables. PVC, ECA and BCA showed a clear influence of cell viability when the lipophilic extraction medium is used. The examination of a lipophilic extraction medium can only be carried out by the agar overlay method, because direct contact of an oily medium would damage the cells. Thus, the agar overlay is a suitable method to classify hydrophilic or lipophilic components as responsible for a cytotoxic reaction. It is a rapid and simple assay, but does not allow a quantitative evaluation of the cytotoxicity. Hence, a combination of the agar overlay method with other in vitro cell culture methods for assessing biocompatibility is important.

4.4. Indirect contact method

In the indirect contact assay, the inserts prevent mechanical damage of the cell monolayer. The polymer tablets are in contact with the cell culture medium during the entire incubation period of 4 days. Leachables and degradation products can diffuse to the cell layer. Evaluating the cell growth after indirect exposure of the test materials RG 503 and RG 505 did not show any effect. In contrast, PCAs decreased the cell proliferation to the same extent as the toxic PVC.

4.5. Direct contact method

The SEM studies are in agreement with the results of the extraction method, agar overlay method and indirect contact method, since good cell spreading is shown by the PLGA films, indicating that these materials are not toxic to the fibroblasts. The PCAs contrast with PLGA, as no cell spreading can be observed corresponding to the investigations of other groups [12,35]. Neupert [34] described adherence, spreading and even proliferation of rat fibroblasts RL19 on polymerized BCA films. We cannot confirm these findings since our investigations showed very little adherence of L929 cells without spreading. Lack of cell adherence and spreading could not always be interpreted as cytotoxicity because surface properties of the material play an important role, but in combination with the extraction method, agar overlay and indirect contact, these results support the toxic effect of PCAs.

5. Conclusions

To determine the biocompatibility of a material, it is necessary to carry out a variety of different screening methods. The cytotoxicity of biomaterials is only one aspect of biocompatibility, which can be assessed by inexpensive in vitro screening tests using cell culture models. Using different polymer preparations recommended by the International Standard Organization, we found PLGA to be very well tolerated by the cultured L929 cells in all assays performed, whereas PCAs showed toxic effects. Toxicity graduation follows the alkyl chain length of the PCAs with MCA being the most toxic compound tested. All polymer preparation methods demonstrated the cytotoxicity of the PCAs, but only the extraction method evaluated by the MTT assay showed a clear graduation of in vitro cytotoxicity and an estimation of IC_{50} values. These in vitro results correspond to published data on tissue compatibility in animal models. In vitro cell culture methods for evaluation of biocompatibility cannot substitute testing of local tolerance in animals. They are, however, quite useful as a screening test for ranking different biomaterials.

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