

## A highly sensitive cell assay for validation of purification regimes of alginates

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### Abstract

Among the hydrogels used for microencapsulation of cells and tissues, alginate has been and will continue to be one of the most important biomaterials. A mandatory requirement for clinical immunisolated transplantations is the reproducible production of biocompatible alginate. As shown here for alginates extracted from freshly collected algal stipes, the current assays used for validation of the quality of the alginate are not sufficient to screen for impurities arising from spores of gram-positive bacteria (and related contaminants). To assess the quality of alginate, we have developed a cell assay based on the induction of apoptosis in Jurkat cells. This assay allows in combination with the “modified mixed lymphocyte” assay a rapid and sensitive screening for any fibrosis-inducing impurities in alginate samples (even during the purification regime) as demonstrated by transplantation experiments performed in parallel with BB rats (exhibiting an elevated macrophage activity).

The results clearly demonstrate that the quality of the input algal material is of key relevance for the production of transplantation-grade alginate.

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### 1. Introduction

Mammalian cell microencapsulation technology has attracted considerable interest in recent years because transplantation of encapsulated cells or tissues allows to correct hormone (or other factors) deficiency diseases at the cellular level without the need of immunosuppression [1,2]. To achieve this goal, a microcapsule must be developed that meets a set of stringent demands [3–5]. The difficulty of designing microcapsules with optimum clinical features is to adjust the numerous capsule parameters (such as biocompatibility, size, permeability, mechanical strength, surface topography, etc.) and host-related factors properly. Seemingly minor modifications

of one capsule parameter can have an enormous impact on the other capsule parameters resulting in ultimately graft failure.

Alginate-based microencapsulation is currently a favoured approach because animal studies and small-scale clinical trials have shown [1–10] that the requirements for long-term immunoisolation and simultaneous maintenance of transplant function can most likely be fulfilled by this hydrogel. Formation of alginate-based immunisolating capsules requires alginate of extreme purity, high viscosity and standardised mannuronic/guluronic acid composition. Purification of crude commercial alginates has the disadvantage that many mitogenic, cytotoxic and apoptosis-inducing impurities (introduced partly by the manufacturing process from contaminated algal material) must be removed in a very time- and manpower-consuming manner [11]. Further and very important drawbacks of commercial alginates as input source for clinical-grade alginate are the variation in the mannuronic/guluronic acid composition

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and that cleavage of the polymeric mannuronic and guluronic acid chains occurs during the extraction process resulting in alginates of relatively low viscosity (about 1–5 mPa s; 0.1% solution in distilled water). However, work in our laboratory has given evidence that clinical-grade alginates of ultra-high viscosity (UHV) (about 20–30 mPa s) have transplantation properties superior to low-viscosity (LV) alginates [5]. Such UHV-alginates can be obtained by extraction of the alginate from fresh, peeled stipes of brown algae harvested directly from the sea. Stipes as starting material also allow to simplify the purification process considerably thus enabling large-scale production at relatively low costs. However, there is a bulk of evidence that the purity of the alginate depends strongly on the quality and age of the stipes.

Before regulatory approval of UHV-alginate can be granted, we need fast and sensitive assays to validate the purification process and to assure the reproducible quality of the product because sea water pollution as well as microbial, viral and other factors can interfere with the starting material and thus with the purification regime [5,12–15]. Current routine analytical tests comprise measurements of endotoxin, protein and polyphenolic-like substances [5,12]. Measurements in combination with implantation studies have, however, shown [5,12] that these tests are necessary, but not sufficient to exclude immunological and inflammatory reactions against remaining contaminants and bacterial components in the alginate under in vivo conditions.

In this research, we present an apoptosis assay for assessment of biocompatibility of alginates. Performance of this assay needs only a few days. When used together with a “modified mixed lymphocyte” test [12] the assay is as sensitive as 4-week implantation of empty microcapsules in spontaneously diabetic BB rats. BB rats are the most stringent small animal model to date, presumably because of their elevated macrophage activity [5,8,12]. As shown recently [5,12], results observed in these rodents can easily be extrapolated to transplantation in human beings without any ethical problems. Thus, it appears that a large part of time-consuming, expensive and sometimes very variable animal studies can be avoided when this assay is routinely used for manufacturing alginate for clinical trials. The apoptosis assay is further an important tool for improvement of the quality of the alginate. This seems to be mandatory because of the finding reported here that purified alginate can frequently be contaminated by spores of gram-positive bacteria if the harvesting process of the algae and the subsequent extraction process is not performed extremely carefully. To our knowledge, this kind of contamination has not been addressed by workers in the field so far.

## 2. Materials and methods

### 2.1. Alginates

For the production of UHV-alginate fresh stipes of *Laminaria pallida* (southern Africa, denoted by the subscript “Lam”) and *Lessonia nigrescens* (Chile, denoted by the subscript “Les”) were used as starting material for extraction and purification of alginates. Immediately after harvesting from the sea or from the beach the outer approximately 1–2 mm layer of the stipes was removed; the stipes were then washed, cut into small pieces and dried for about 8 h from 20°C to 35°C outdoors. After transportation of the dried material to Germany, the material was vacuum-sealed.

Alginate extracted from stipes harvested in the sea was denoted according to the collection time (March = 3 and October = 10), i.e. UHV<sub>Lam3</sub>, UHV<sub>Lam10</sub> and UHV<sub>Les3</sub>. In the case of *L. pallida* freshly looking material collected at the beach or mixtures of fresh beach and sea algae were also used for alginate extraction. This alginate was denoted with the additional subscripts “b” and “bs” (i.e. UHV<sub>Lam10,b</sub> and UHV<sub>Lam10,bs</sub>), respectively.

For control, the LV-alginates Manugel GHB and GMB (NutraSweet Kelco, Giran, UK) were used without any further purification. The viscosity of a 0.1% w/v commercial alginate solution in distilled water was with 1–5 mPa s significantly lower than the corresponding value of 20–30 mPa s for the UHV-alginates [5,12,16].

### 2.2. Extraction and purification of UHV-alginates

Production of UHV-alginates was performed as described in Refs. [5,12,16]. Briefly, the algal material was extracted with 50 mM EDTA, the solution filtered and the alginate precipitated with ethanol (37.5% v/v) under injection of air. The precipitate was dissolved in 0.5 M KCl solution and subjected to two ethanol precipitation steps again. Sometimes, a dialysis step was introduced before the third precipitation. The entire process was performed at room temperature in order to avoid depolymerisation at elevated temperatures. Finally, the white precipitate was sterilised with ethanol, dried under sterile conditions and stored at 4°C.

In order to remove contaminants from alginate extracted from *L. nigrescens*, it was necessary to filter the EDTA extraction solution through charcoal-containing filters or to treat the filtered solution with charcoal. In this case, it was also necessary to re-hydrate the dried raw material before EDTA treatment.

In some experiments with UHV<sub>Lam10,b</sub>, the dry algal material was pre-incubated for up to 1 or 5 days in distilled water containing 100 µg/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B to

eliminate bacteria and yeasts, respectively (PAA, Linz, Austria).

### 2.3. Analytical assays

Impurities of proteins and polyphenolic-like compounds in the purified UHV-alginate lots were determined by fluorimetric measurements using a LS50 luminescence spectrometer (Perkin Elmer, Beaconsfield, UK; excitation/emission 270 nm/300–350 nm and 366 nm/380–550 nm, respectively; [16,17]). Contaminations with endotoxins were proved by using the limulus-lysate assay (0.25% w/v alginate in sterilised 0.9% NaCl solution). When the level of the above contaminants was found to be negligible or below the regulatory threshold [18], the alginate samples were additionally analysed by NMR spectroscopy in order to screen EDTA and ethanol traces as well as for other impurities and metabolites [19,20]. To this end, the dried alginate was dissolved in deuterated water ( $D_2O$ ) with an isotopic purity of 99.96%  $^2H$ -containing TSP- $d_4$  (trimethylsilyl-tetradeutero-propionate, sodium salt) as frequency and concentration standards [21]. The final concentration of alginate and TSP- $d_4$  was 5 mg/ml and 0.25 mM, respectively. Chemicals were of highest commercially available quality (Fluka, Neu-Ulm, Germany) and were used without any further purification.

The NMR measurements were performed on a Bruker DRX-600 spectrometer operating at 600.13 MHz for  $^1H$  using a 5 mm “direct”, tuneable probe (5 mm BBO). Alginate spectra of 0.5 ml samples were recorded at 50°C with or without water suppression. Water suppression was achieved by pre-saturation, i.e. a saturation pulse at the water resonance [22]. Sixty-four free induction decays were acquired with a total delay of 8 s between two consecutive pulses in order to allow complete relaxation. The 90° excitation pulses (about 7  $\mu$ s) were used. The spectra were recorded with a spectral width of 6000 Hz corresponding to about 10 ppm and 16 K data points. No window functions were used prior to Fourier transformation.

Chemical shifts were referenced to the trimethylsilyl resonance of the internal TSP- $d_4$  (at 0.00 ppm). Resonances were identified by their known chemical shifts and by subsequent enhancement after addition of a small amount of the suspected pure compounds. This was especially helpful when singlets (e.g. arising from EDTA) had to be assigned.

### 2.4. Microcapsule formation

For microcapsule formation, UHV-alginate was dissolved in sterile, endotoxin-free 0.9% NaCl solution. The alginate concentration was adjusted to 1.0%, except that in two animal implantation studies with UHV<sub>Les3</sub> a concentration of 0.7% was used. In part of the

experiments, 10% fetal calf serum (FCS; PAA, Linz, Austria) was additionally added to the solution in order to improve the stability of the microcapsules under in vivo conditions [23]. Because the results did not differ significantly from those obtained by the standard procedure, the data were pooled.

Homogeneous microcapsules were performed by using a two-channel air-jet droplet generator as described in Refs. [16,24]. For cross-linking of the alginate, the microcapsules were dropped into a 115 mM NaCl solution containing 20 mM  $BaCl_2$  and 5 mM histidine (pH 7). The osmolality of this solution was adjusted to 290 mOsm by adding appropriate amounts of NaCl. The microcapsules were removed after 15 min and washed three times with 0.9% NaCl solution. Then, the microcapsules were incubated in a 6 mM  $Na_2SO_4$  solution (adjusted by NaCl to 290 mOsm) for 30 min at 37°C in order to precipitate excessive  $Ba^{2+}$  ions [5,12,23]. Finally, the microcapsules were washed twice with a 0.9% NaCl solution.

For microcapsules made up of commercial LV-alginates the same protocol was used, except that the concentration of alginate was adjusted to 2% and that the  $Na_2SO_4$  treatment was omitted.

### 2.5. Implantation

Empty  $Ba^{2+}$  cross-linked microcapsules were implanted beneath the kidney capsules of spontaneously diabetic BB rats [5,12]. After 3 weeks, the implants were removed and processed for histological analysis. The serial sections of the explanted kidney were stained with haematoxylin/eosin and then examined with a light microscope. The histological evaluation of the code-numbered implants was performed by Prof. Hahn (Institute of Pathology, Karlsburg, Germany), Dr. Julius (Julius-Institute, Biomedical Investigations GmbH, Großwallstadt, Germany) and other experts not involved in this study.

### 2.6. Lymphocyte activation and proliferation assay

Lymphocytes were isolated from spleens of male, 6–8-week old Balb/c mice (Charles River Wiga, Sulzfeld, Germany). Spleens were microdissected in segments. Each segment was placed immediately in 25 ml complete growth medium (CGM) consisting of phenol red containing RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 2 mM sodium pyruvate, 1  $\times$  non-essential amino acids, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin (purchased from PAA, Linz, Austria) and 50  $\mu$ M 2-mercaptoethanol (Merck, Darmstadt, Germany). After removal of adipose tissue, the spleen fragments were mashed and cleaned by using a 100- $\mu$ m cell strainer (Becton Dickinson Labware, Franklin Lakes, USA). Then, the cell suspension was

centrifuged at 180g for 10 min. The erythrocyte-containing supernatant was discarded and the pellet was re-suspended in 25 ml of a 0.8% NH<sub>4</sub>Cl solution (Merck, Darmstadt, Germany) and kept for 10 min at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. After centrifugation the supernatant was discarded, the cells were re-suspended in 25 ml of the 0.8% NH<sub>4</sub>Cl-solution and the cell suspension was kept for 10 min at 37°C in a 5% CO<sub>2</sub> humidified atmosphere again. Later the cells were washed with CGM three times.

For the proliferation assay, the lymphocytes were re-suspended in CGM and the suspension density was adjusted to  $1 \times 10^6$  cells/ml. The sensitivity of the proliferation assay for detection of mitogenic impurities in the (code-numbered) alginate lots could greatly be enhanced when the lymphocytes were co-activated with lipopolysaccharide (LPS; Sigma, Deisenhofen, Germany) [12,16]. To this end, 3 µl of a 1 mg/ml LPS stock solution (dissolved in distilled water) were added to 300 µl of the suspension. Then the suspension was transferred into three wells of a 96-well plate (TPP, Trasadingen, Switzerland). Exactly 20 µl of a 0.25% alginate solution (dissolved in 0.9% NaCl solution) was added per well. The final concentrations of LPS and alginate were 8 µg/ml and 0.04% w/v, respectively. Cells were kept for 3 days at 37°C in a 5% CO<sub>2</sub> humidified atmosphere before the ratio of dead cells/debris and non-stimulated cells (diameter <8 µm) to viable, proliferating cells (diameter >9 µm) was determined by using an electronic size analyser (CASY-1 instrument, Schärfe System, Reutlingen, Germany) [25]. The area beneath the size distribution of the proliferating cells in relation to the area of the size distribution of the control cells stimulated only with LPS was defined as the mitogenic index and was taken as an estimate for the concentration of mitogenic contaminants in the alginate. Calculation of the areas was performed by using Microsoft Excel.

### 2.7. Apoptosis assay

Human Jurkat T-cell leukemia cells (ACC 282, DSMZ, Heidelberg, Germany) were cultured in Jurkat medium consisting of phenol red containing RPMI 1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 U/ml streptomycin (PAA, Linz, Austria). The cell culture was kept at 37°C in a humidified atmosphere enriched with 5% CO<sub>2</sub>. Every 2–3 days, cells were transferred into culture flasks with fresh Jurkat medium. Cells were passaged at least 20 times before use. For the apoptosis assay cells from the logarithmic growth phase were taken. Cells were centrifuged at 180g for 10 min. The pellet was re-suspended in 10 ml Jurkat medium to which 2 ml of a 0.5% w/v (code numbered) alginate solution (dissolved in 0.9% NaCl solution) were added. The suspension

density was adjusted to  $1.7 \times 10^5$  cells/ml. The suspension was transferred into three wells of a 12-well plate. Cells were grown for about 2 days at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. For negative and positive controls, cells were treated in the same way except that alginate was omitted. For positive control camptothecin, a specific apoptosis-inducing reagent [26–28] was used (final concentration in the Jurkat medium 0.75 µg/ml; Sigma, Deisenhofen, Germany).

Viability of the cells before and after 2-d incubation was determined by using 0.05% w/v trypan blue (Biochrom, Berlin, Germany). Additionally, the percentage of the G<sub>0</sub>/G<sub>1</sub>-, S- and G<sub>2</sub>/M-phases was determined by propidium iodide staining of saponin-lysed cells (final concentration 2 mg/ml; Sigma, Deisenhofen, Germany) and subsequent flow cytometry analysis (Coulter, Krefeld, Germany) as described in detail elsewhere [29]. Blocking of the S-phase of the Jurkat cells by addition of camptothecin was determined in the same way. Data were analysed by using System II<sup>TM</sup> software (Coulter, Krefeld, Germany) and WinMDI flow cytometry software (TSRI, La Jolla, USA).

For detection of apoptosis by flow cytometry analysis, the MitoCapture Mitochondrial Apoptosis Detection Kit (BioCat, Heidelberg, Germany) was used. This kit consists of an incubation buffer and of a solution containing the fluorescence cyanine dye JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). This dye accumulates in the mitochondrial matrix of healthy cells under the influence of the membrane potential and forms aggregates [30–36]. When excited with 488 nm, the JC-1 aggregates emit bright red fluorescence  $590 \pm 42$  nm (FL-2). In the case of apoptosis, a shift to lower red fluorescence is observed accompanied by an increase in green fluorescence  $530 \pm 30$  nm (FL-1). This shift is induced by a decrease of the mitochondrial membrane potential.

Screening experiments showed that the standard protocol of the manufacturer required some modifications in order to increase the fluorescence signals in the presence of alginate. Best results were obtained by using the following protocol. An aliquot of 2 ml cell suspension was centrifuged at 180g for 10 min and re-suspended in 1 ml incubation buffer to which 0.5 µl of JC-1 was added. Then the cells were incubated for 20 min at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. After centrifugation at 180g for 10 min, the supernatant was discarded and the pellet re-suspended in 1 ml phosphate buffered saline (PAA, Linz, Austria). The JC-1 labelled cells were immediately analysed by flow cytometry analysis. After gating out small-sized (including cellular and non-cellular) debris 10,000 events were collected for each analysis. Based on forward and side scatter, cells were gated into high and low fluorescent FL-1 and FL-2 populations, i.e. in apoptotic and healthy cells. The percentage ratio of green fluorescent

cells in the alginate-treated cells to green fluorescent cells in the untreated cells was defined as apoptotic index and was taken as a measure for apoptotic contaminants in the alginates.

### 2.8. Statistical analysis

In order to estimate the statistical significance of the results of the proliferation and apoptosis assay a two-population student *t*-test was used. To this end, means  $\pm$  standard errors (SE) of mitogenic and apoptotic indices, respectively, were calculated and tested whether or not the population means were equal or significantly different.  $P < 0.05$  was considered as statistically significant.

## 3. Results

If not stated otherwise, algal material was used that was not pre-treated with antibiotics. When purified alginate was added to complete growth media containing 10% fetal calf serum or 1% human serum albumin, bacterial growth could occur indicating that spores were apparently not completely eliminated during the production process. Contamination was quite variable and apparently varied with weather conditions and season of collection. Age and quality of the stipes also seemed to play an important role as suggested by investigations of very carefully selected material. On average, alginate extracted from algae harvested in the sea contained significantly less bacterial spores than alginate extracted from freshly looking algae collected at the beach. Some “sea alginate” samples particularly when extracted from young algae carefully selected were completely free of spores. Spores could largely be removed both from “sea” and “beach” alginate by treatment of the algal material with antibiotics before alginate extraction.

The use of antibiotic-treated and untreated material appeared reasonable in order to prove the potent activity of the new cellular assay in comparison to the animal studies and to demonstrate further the unsatisfactory results obtained by current analytical assays employed for quality control.

### 3.1. Analytical assays

Fluorescence spectroscopy revealed that the level of polyphenolic-like compounds and proteins in UHV-alginates was quite low in contrast to commercial LV-alginates (Figs. 1A and B). NMR spectroscopy confirmed these results. In Fig. 1C, typical NMR spectra of commercial LV-alginate GHB (curves 1 and 2) and UHV<sub>Lam10,b</sub>-alginate (curves 3 and 4) are depicted. Spectra 2 and 4 in Fig. 1C represent the single pulse spectra obtained by the application of a single 90° pulse

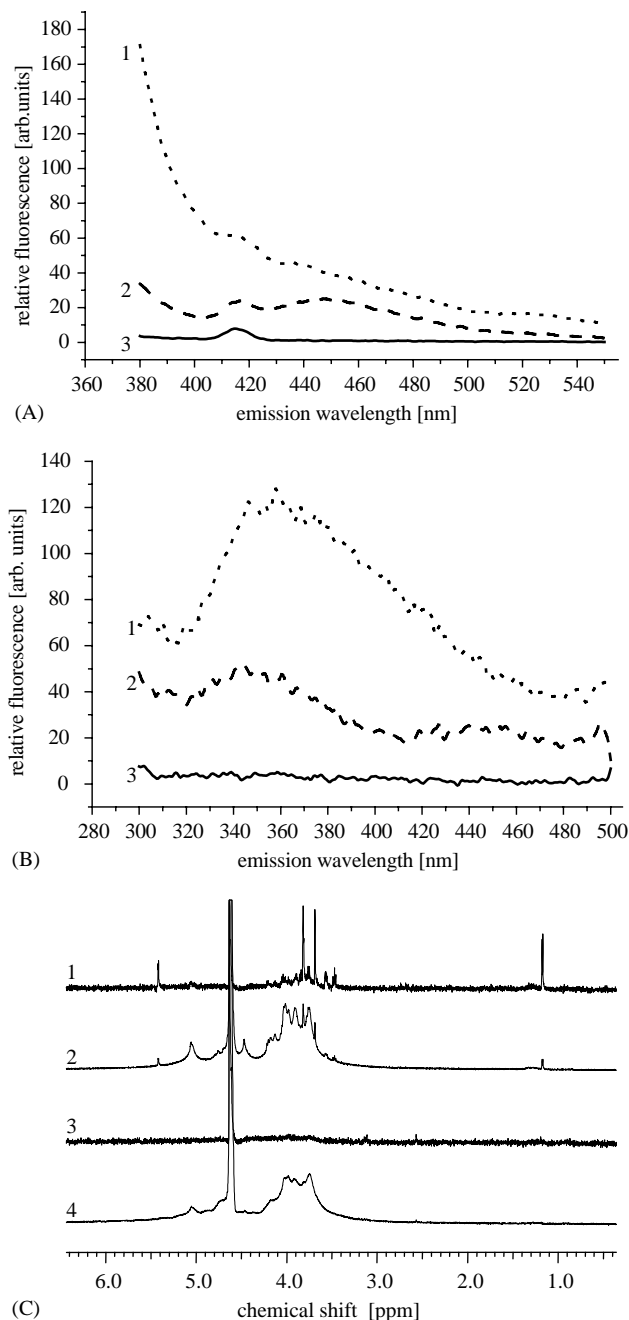


Fig. 1. Determination of polyphenolic-like, protein and other impurities in alginates by fluorescence (A,B) and <sup>1</sup>H NMR (C) spectroscopy. (A) polyphenolic-like contaminants (excitation/emission 366 nm/380–550 nm); (B) protein contaminants (excitation/emission 270 nm/300–500 nm); curve 1 commercial LV-alginate, curve 2 UHV<sub>Lam10,b</sub>-alginate and curve 3 control (distilled water); and (C) 600.13 MHz <sup>1</sup>H NMR spectra of commercial LV-alginate (spectra 1 and 2) and of UHV<sub>Lam10,b</sub> alginate (spectra 3 and 4) samples dissolved in D<sub>2</sub>O at a concentration of 5 mg/ml (recordings performed at 37°C). Spectra (2) and (4) correspond to the single pulse spectra, whereas (1) and (3) represent spectra recorded under conditions in which the water resonance was saturated by an additional pulse. For more details, see text.

for excitation. The sharp peak at about 4.6 ppm could be assigned to residual water and the broad peaks between 3.5 and 4.3 ppm to alginate. It was obvious that the

commercial LV-alginate spectrum exhibited several more peaks than the UHV<sub>Lam10,b</sub>-alginate spectrum. When the spectra were recorded under saturation of the water resonance frequency (i.e. by so-called pre-saturation; see above), the intensity of the water resonance was attenuated (curves 1 and 3). Surprisingly, the alginate resonance was also suppressed. The reason for this is not completely understood. It was presumably caused by saturation transfer from the water to the alginate protons [37].

Due to the suppression of the water and alginate resonances upon application of the water saturation pulse sequence, a number of sharp resonances became clearly visible in the spectrum of commercial LV-alginate (curve 1) that were not seen in the corresponding spectrum of UHV<sub>Lam10,b</sub>-alginate (curve 3). These resonances arose most probably from contaminants. The detailed chemical structure of all these impurities is not known. However, some of the less intense resonances were most likely caused by alginate oligomers [20] or by other carbohydrates presented in brown algae [19]. This was suggested by the resonances at 5.42 and 5.05 ppm which were in the typical spectral range of anomeric protons.

The very intense resonances at 3.82 and 3.68 ppm in the spectrum of the commercial LV-alginate were most likely caused by EDTA (which is commonly used for alginate extraction from brown algae; see also above and Ref. [38]). Unfortunately, the resonance frequencies of EDTA were influenced by many parameters, e.g. the pH and the ion concentration and, therefore, unambiguous assignment was not possible if only proton NMR spectra were considered. The theoretically expected intensity ratio of 1:2 between both resonances of the EDTA was not exactly found. However, this was not surprising since it is known that upon binding of bivalent ions like Ca<sup>2+</sup> or Mg<sup>2+</sup>, a change of the chemical shift and of the relative intensities of the EDTA protons is induced. The poor signal-to-noise ratio of the spectra recorded under pre-saturation conditions suggested that the concentration of contaminants and oligosaccharides in commercial LV-alginate was < 100 µM. Since sharp resonances were not found in the pre-saturation spectrum of UHV<sub>Lam10,b</sub>-alginate (curve 3), it was obvious that the level of impurities (including EDTA) must be extremely small in alginates extracted from freshly collected, peeled algal stipes.

### 3.2. Animal studies

Despite the high analytical quality of UHV-alginates, fibrotic reactions (together with inflammatory reactions) were observed when empty microcapsules were implanted beneath the kidney capsule of BB rats. For implantation, alginates were used which were pre-analysed for endotoxins. Determination of endotoxins

in UHV-alginates extracted from algae not treated with antibiotics showed that the concentration was usually < 2.5 EU/ml, i.e. much less than that requested for medical approval (10 EU/ml). This was even the case for UHV<sub>Lam10,b</sub> that was quite frequently contaminated with bacteria and that induced generally significant fibrotic reactions when implanted under the kidney capsule of BB rats (see below). This result suggested that the spores that were apparently present in purified alginate extracted from untreated algae must arise from gram-positive bacteria. As expected, the immunological responses depended on the season and on the collection site (beach or sea). As shown in Fig. 2, these could range from no fibrotic reaction (a) to strong fibrotic overgrowth (f). Microcapsules made up of the same material and implanted simultaneously beneath the right- and left-kidney capsule could also show quite significant variations. Even microcapsules implanted under the same kidney capsule induced sometimes a different fibrotic response. While two microcapsules could be surrounded by a relatively thick fibrotic layer, the third microcapsule could be free of fibrotic overgrowth (data not shown). Quantification of fibrosis was, therefore, quite difficult. However, in order to correlate the broad spectrum of fibrotic responses in the BB rats with the data of the apoptosis/proliferation assay, it appeared useful to characterise the immunoreactivity of the various alginate lots by definition of five reaction types. As indicated in Fig. 2b, very weak fibrotic overgrowth was denoted with the sign +. Such foreign body reactions apparently do not prevent nutrient and oxygen exchange between encapsulated cells and their environment. Rather, such a reaction could be advantageous under some circumstances because microcapsule breakage due to shear forces (e.g. in the muscle) and movement from the transplantation site will be minimised. Weak fibrosis of alginate microcapsules denoted with the sign ++ (Fig. 2c) is obviously not satisfactory but can yet be classified under some circumstances as relatively biocompatible provided that optimal nutrient supply is guaranteed by vascularisation of the microcapsules. In contrast, alginate microcapsules provoking moderate (+++), intense (++++) and strong (++++) fibrosis are apparently improper for immunoisolation of transplants (Figs. 2d–f).

According to the above classification, commercial LV-alginates were always strongly fibrotic (++++) as already found previously in Ref. [11] (see also Fig. 2f). Conversely, UHV-alginates subjected to the extraction and purification process of the starting algal material provoked on average considerably less fibrotic overgrowth. The results of 68 implantation studies with UHV-alginates are listed in Table 1. Inspection of the data shows that alginates extracted from fresh algae collected at the beach (UHV<sub>Lam10,b</sub>) were quite reactive compared with the other UHV-alginates. Only one

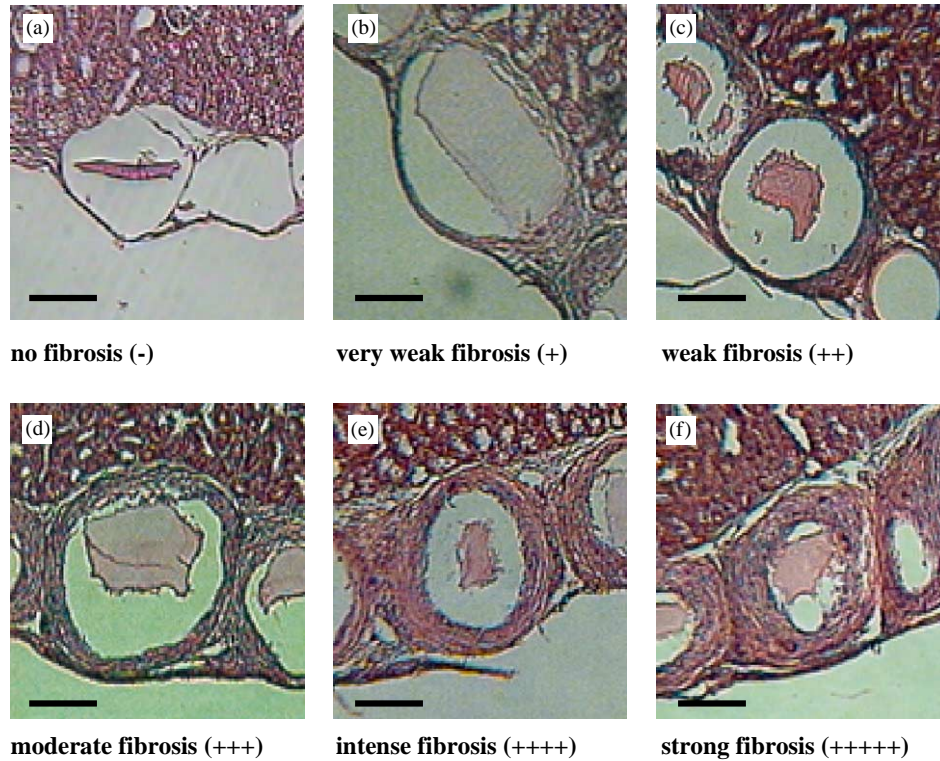


Fig. 2. Alginate microcapsules from UHV-alginates were implanted beneath the kidney capsules of spontaneously diabetic BB rats. After 3 weeks, the implants were retrieved and processed for histological analysis (haematoxylin/eosin staining). The different degrees of fibrosis were assigned with “+” signs ranging from zero for no fibrosis (a) to five “+” signs for strong fibrosis (f). Bar = 150 μm.

Table 1  
Fibrotic reactions induced by microcapsules made up of UHV-alginates and implanted beneath the kidney capsules of spontaneously diabetic BB rats<sup>a</sup>

| UHV-alginates           | - | + | ++ | +++ | ++++ | +++++ | n  |
|-------------------------|---|---|----|-----|------|-------|----|
| UHV <sub>Lam10</sub>    | 0 | 3 | 6  | 6   | 3    | 0     | 18 |
| UHV <sub>Les3</sub>     | 0 | 6 | 5  | 0   | 0    | 0     | 11 |
| UHV <sub>Lam3</sub>     | 1 | 8 | 4  | 2   | 0    | 0     | 15 |
| UHV <sub>Lam10,bs</sub> | 0 | 1 | 4  | 5   | 1    | 1     | 12 |
| UHV <sub>Lam10,b</sub>  | 0 | 0 | 1  | 3   | 7    | 1     | 12 |

<sup>a</sup>For evaluation see Fig. 2.

implant out of 12 showed fibrotic reactions that met the criteria for biocompatibility. When alginate extracted from mixed beach and sea material was used (UHV<sub>Lam10,bs</sub>), the number of biocompatible microcapsules increased significantly (Table 1). Best results were obtained with alginates extracted from pure sea material (UHV<sub>Lam3</sub>, UHV<sub>Lam10</sub> and UHV<sub>Les3</sub>). However, also in this case pronounced differences between the three alginates could be seen. UHV<sub>Les3</sub>-alginate extracted from *L. nigrescens* showed very good and, in particular, very reproducible results; fibrotic overgrowth ranged only from + to ++. Alginate extracted from *L. pallida* collected during March (UHV<sub>Lam3</sub>) yielded extremely

good results. Nine implants showed no or a very weak fibrotic reaction and four implants only a weak fibrotic overgrowth that could be assigned with ++. However, in two cases, the fibrotic overgrowth was out of the defined range of biocompatibility. Interestingly, 50% of the microcapsules made from alginate extracted from *L. pallida* harvested from the sea during October showed moderate (+++) up to intense reactions (++++).

In order to compare the quite varying results of the animal studies with the data of the apoptosis and proliferation assays reported below, it seemed useful to introduce a fibrotic index for characterisation of the UHV-alginates. To this end, the value “0” was assigned for no fibrosis (–) and accordingly values “1–5” for very weak (+) up to strong fibrosis (+++++). The mean ± SE of the fibrotic values obtained for a given UHV-alginate was taken as a measure for averaged frequency of fibrosis (see Table 2).

Evidence that the immunogenicity resulted to a large extent from contamination of the algae with gram-positive bacterial spores was obtained when alginates extracted from antibiotic-treated input material were implanted. For example, in the case of UHV<sub>Lam10,b</sub>, the foreign body reactions observed after 3-week implantation were comparable to that recorded with high-quality samples of UHV<sub>Les3</sub> and UHV<sub>Lam3</sub> (up to ++). As shown in the following, mitogenic impurities could be

Table 2  
Fibrotic reactions versus apoptotic and mitogenic indices<sup>a</sup>

|                 | Controls without alginate     |                 |                 | Commercial LV- alginate           |                |                |                |                |                                   |  | UHV-alginates |  |  |
|-----------------|-------------------------------|-----------------|-----------------|-----------------------------------|----------------|----------------|----------------|----------------|-----------------------------------|--|---------------|--|--|
|                 | Negative control <sup>b</sup> | + LPS           | + Camptothecin  | GHB                               | Lam10          | Less3          | Lam3           | Lam10,bs       | Lam10,b                           |  |               |  |  |
| Apoptotic index | 1.00±0.00 (63)                | 1.20 (2)        | 12.07±0.38 (63) | 0.79±0.03 (44)                    | 1.13±0.06 (25) | 1.13±0.06 (19) | 1.17±0.03 (19) | 1.15±0.06 (23) | <b>1.55±0.06<sup>c</sup></b> (37) |  |               |  |  |
| Mitogenic index | 0.21±0.04 (9)                 | 1.00±0.00 (9)   | 0.51 (2)        | <b>2.07±0.19<sup>c</sup></b> (8)  | 0.57±0.10 (9)  | 0.66±0.15 (5)  | 0.23±0.01 (3)  | 0.74±0.12 (12) | 0.57±0.04 (23)                    |  |               |  |  |
| Fibrotic index  | ND <sup>d</sup>               | ND <sup>d</sup> | ND <sup>d</sup> | <b>4.80±0.13<sup>c</sup></b> (10) | 2.50±0.23 (18) | 1.45±0.16 (11) | 1.47±0.21 (15) | 2.75±0.31 (12) | 3.66±0.23 (12)                    |  |               |  |  |

<sup>a</sup>Data are given as mean ± standard error; number of independent experiments in brackets.

<sup>b</sup>Negative controls of the apoptosis and proliferation assays were not incubated with LPS and camptothecin, respectively.

<sup>c</sup>Bold letters indicate experiments in which the apoptotic, mitogenic, and fibrotic index, respectively, assumed maximum values.

<sup>d</sup>ND: not determined.

excluded as the main reason for fibrotic reactions induced by UHV-alginates.

### 3.3. Proliferation assay

Fig. 3 shows typical electronic size distribution measurements of murine lymphocytes co-stimulated by LV- and UHV-alginates, respectively, together with LPS. As indicated, cell debris/non-stimulated cells could be clearly distinguished from activated lymphocytes. Comparison of the size distributions of the control lymphocytes (stimulated only with LPS; curve b) with those measured in the presence of commercial LV-alginates (curve c) shows that these alginates were highly mitogenic. The mitogenic index was on average  $2.07 \pm 0.53$  ( $n = 8$ ). It is obvious that the pronounced mitogenicity of commercial alginates corresponded well with the extremely heavy fibrotic reaction in BB rats.

In contrast, UHV-alginates were non-mitogenic (curves f and g). Their mitogenic index was even smaller than unity indicating that these alginates suppressed the mitogenic activity of LPS. The values of the mitogenic index ranged between 0.23 and 0.74 (Table 2). The differences between the mitogenic indices of the various UHV-alginates were, however, statistically not significant ( $P < 0.05$ ). This means that it was not possible to distinguish between the “sea alginate” UHV<sub>Lam10</sub> and the “beach alginate” UHV<sub>Lam10,b</sub> which exhibited quite large differences in their fibrotic responses in the animal (see above) corroborating the view that contamination

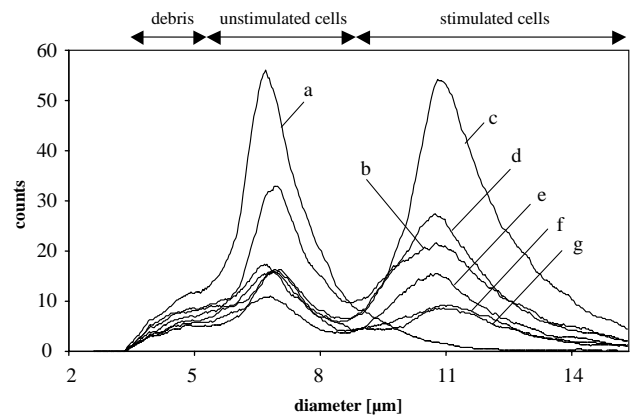


Fig. 3. Proliferation assay for detection of mitogenic impurities of alginates. The assay is based on the activation and proliferation of murine lymphocytes isolated from male Balb/c mice. Samples were co-activated simultaneously with LPS and alginate (0.04% w/v). After 3 days, unstimulated ( $< 8 \mu\text{m}$ ) and stimulated cells ( $> 9 \mu\text{m}$ ) were measured by means of an electronic cell counter. (a) Unstimulated cells without LPS and alginate (negative control); (b) cells only stimulated with LPS (positive control); (c) commercial LV-alginate; (d) beach alginate after first precipitation step; (e) beach alginate after second precipitation step; (f) UHV<sub>Lam10,b</sub>-alginate; and (g) UHV<sub>Lam10</sub>-alginate.

with gram-positive bacterial spores is mainly responsible for immunogenicity of the hydrogels.

Measurements of the mitogenic index of alginate samples taken after each ethanol precipitation step (Fig. 3 curves d and e) of beach algae demonstrated that most of the mitogenic contaminants were removed after the second precipitation step. Thus, three ethanol precipitation steps seem to be sufficient for removal of mitogenic contaminants. This is an important prerequisite for interpretation of apoptosis assay data which can apparently be affected, but differently by mitogenic contaminants as well as by related components related with bacterial spores.

Pre-treatment of the beach algae with antibiotics before subjection to the complete extraction and purification process induced a mitogenic index that was comparable to the level of alginate extracted from algae harvested in the sea ( $0.61 \pm 0.09$ ;  $n = 4$ ).

### 3.4. Apoptosis assay

Screening experiments showed that the sensitivity and reproducibility of the apoptosis assay depended strongly on the number of passages and cell viability. Cell suspensions selected after the 20th passage exhibited optimal features for the detection of apoptosis-inducing contaminants in alginates. Only suspensions were used which contained at least 97% viable cells (as revealed by trypan blue staining) and displayed the typical percentage of the G<sub>0</sub>/G<sub>1</sub>-, S- and G<sub>2</sub>/M-phases ( $57 \pm 4$ ,  $21 \pm 3$  and  $22 \pm 3$ , respectively;  $n = 63$ ) when subjected to flow cytometry analysis. Control cells treated with the mitochondria-specific fluorescent dye JC-1 (negative control;  $n = 63$ ) emitted a bright red fluorescence, whereas camptothecin-treated cells (positive control;  $n = 63$ ) exhibited a strong green staining as expected (Fig. 4). In the case of camptothecin-treated cells, only 15% of cells were viable. No cells were detected in G<sub>2</sub>/M-phase of cell cycle and the apoptotic index was extremely high (Table 2).

When commercial LV-alginates were tested, an apoptotic index of 1.0–1.4 was found in eight experiments out of 44. However in 82% of experiments, values between 0.47 and 1.0 were determined, i.e. values which were smaller than those of control cells (not treated with alginate). These findings were presumably due to interferences of the mitogenic impurities in the LV-alginates with apoptotic pathways because UHV-alginates showed apoptotic indices equal or larger than 1.0, except for a very few cases.

The average values of the apoptotic indices of the “sea alginates” UHV<sub>Lam3</sub>, UHV<sub>Lam10</sub> and UHV<sub>Les3</sub> as well as of the “sea/beach alginate” UHV<sub>Lam10,bs</sub> were with 1.13–1.17 (see Table 2) nearly identical and close to the untreated cells. Statistically significant higher values ( $P < 0.05$ ) were found for the “beach alginate”

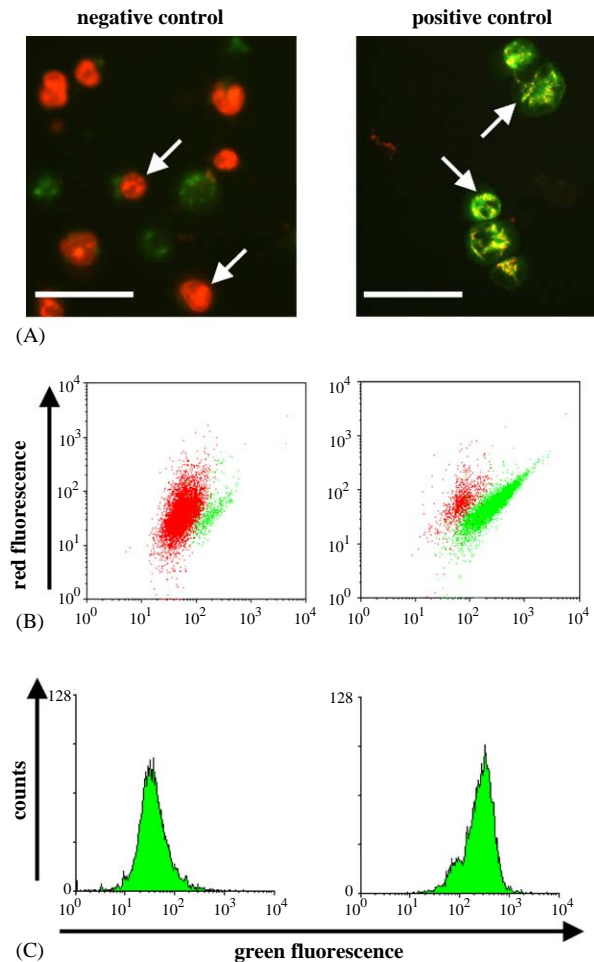


Fig. 4. Jurkat cells untreated (negative control; left panel) or treated with camptothecin ( $0.75 \mu\text{g/ml}$ ; positive control; right panel). Apoptosis of cells was measured by mitochondria-specific fluorescent JC-1 staining. Healthy cells emitted a brightly red fluorescence (left arrows), apoptotic cells emitted green fluorescence (right arrows). (A) Fluorescence microscopy of negative control cells (left) and positive control cells (right). Bar =  $50 \mu\text{m}$ ; (B) flow cytometry analysis of healthy (red marked) and apoptotic (green marked) Jurkat cells; and (C) intensity of green fluorescence of healthy and apoptotic cells. In the case of apoptosis, a shift to an increased green fluorescence is induced because of decrease of the mitochondrial membrane potential.

UHV<sub>Lam10,b</sub> (on average  $1.55 \pm 0.39$ ). This demonstrated that the apoptosis assay was as sensitive as implantation in BB rats.

Measurements of the apoptotic indices of “sea alginates” after the first ethanol precipitation step yielded with  $3.66 \pm 0.57$  ( $n = 4$ ) a relatively high value that dropped to  $1.17 \pm 0.14$  ( $n = 4$ ) after the second ethanol precipitation step. In contrast, the apoptotic index of the “beach alginate” was quite high after the first ethanol precipitation step ( $5.53 \pm 0.10$ ;  $n = 3$ ) and even after the second ethanol precipitation step the value was  $4.71 \pm 0.83$  ( $n = 3$ ). This indicated that three ethanol precipitation steps were not sufficient to remove apoptotic contaminants if they exceed a certain threshold value in the starting alginate material.

The impurities seen by the apoptosis assay could be clearly related to the bacterial spore contamination. When the beach algae were pre-treated with antibiotics before subjection to the complete extraction and purification process the apoptotic index could be reduced to the level of alginate extracted from algae harvested in the sea ( $1.10 \pm 0.15$ ;  $n = 6$ ). In contrast, pre-treatment of the algae with 70% ethanol, 3% formaldehyde or 3% glutardialdehyde had no beneficial effect on the apoptotic index of UHV<sub>Lam10,b</sub>. Similarly, no reduction of the apoptotic index of UHV<sub>Lam10,b</sub> could be observed when the purified alginate was treated with heparinase ( $2.5 \times 10^{-7}$  U/ml) for 24 h at 37°C in order to degrade sulphated polysaccharides commonly present in marine algae [39].

#### 4. Discussion

The purity of the alginate is—among many other things—one obligatory requirement for intra- and also of extracorporeal therapy of human beings. Detailed knowledge of the chemical and physical properties of the (cross-linked) alginates will ensure approval for the use of this hydrogel in biomedical and/or tissue-engineered medical product applications. The ASTM standards for characterisation and testing of alginates are of critical relevance [13,14], but the analytical assays described there and elsewhere [15] apparently do not yield the information required for the prediction of the occurrence of immunological reactions under transplantation conditions. This was demonstrated here by implantation studies of empty microcapsules made up of UHV-alginates cross-linked with Ba<sup>2+</sup> ions. Even though all alginate preparations were of analytical grade and free of endotoxins, a part of them showed intense up to strong pericapsular fibrosis. Fibrotic overgrowth was significantly less and almost absent when stipes were used as starting material for alginate extraction directly harvested from the sea. However, even between these alginate preparations pronounced differences in immunoreactivity were seen. The JC-1 based apoptosis assay reflected these differences in biocompatibility very sensitively. This assay was much more sensitive and also reproducible than the assay based on the phosphorylation of ERK (a component of the MAPK signalling cascade) published recently [40]. Differences in immunoreactivity could be clearly resolved between “sea alginates” (UHV<sub>Lam10</sub>) and “beach alginates” (UHV<sub>Lam10,b</sub>) by using this assay. The “modified mixed lymphocyte” assay frequently used by other authors for testing biocompatibility (literature quoted in [11]) failed completely despite improvements of this assay by co-stimulation with LPS and electronic sizing of the activated lymphocytes [12,15]. However, the modified assay is still valuable in combination with the JC-1

based apoptosis assay because of the detection of mitogenic impurities which facilitates the identification of the apoptosis-inducing contaminants as demonstrated by use of commercial alginates.

As already mentioned in the introduction, kelp can contain not only marine gram-negative, but also gram-positive bacteria and (endo-) spores as well as yeast and fungi [41–44]. Since the extraction and purification processes of alginate comprise only a few steps (mainly EDTA treatment, filtration through 0.2 µm diameter filters and three alcohol precipitations, [5]) it can be expected that the final product is not always free of spores, and/or bacterial components depending—among other things—on the contamination to which the algae were exposed at the collection site and time of harvesting. The treatment of algal input material with antibiotics has given evidence that spores of gram-positive bacteria and/or related (metabolic and structural) components are a very important source for apoptosis-inducing impurities. These were obviously not always eliminated during the purification process.

From work performed in the meantime in the laboratory of U. Hentschel, we know (manuscript in preparation) that alginate extracted from the stipes of brown algae (particularly when collected at the coast of southern Africa) can contain—among other things—substantial amounts of spores of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*, i.e. bacteria commonly occurring in marine environments [41–44]. This bacterial contamination is apparently sensed by the apoptosis assay and obviously also dominates the immunological reactions observed with part of the alginate samples during 3-week implantation. Capsule surface properties apparently played a minor role in the induction of fibrotic overgrowth, otherwise the animal studies and the bioassays would not have yielded consistent results. However, as shown elsewhere [4,5,12], surface topography of the microcapsules is—among other parameters [40]—a critical parameter during long-term transplantation because topographical roughness leads to deposition of cellular material and serum proteins. This seems to favour cell adhesion and induces, in turn, fibrotic overgrowth (for more details, see Ref. [45]). Sulphated polysaccharides or heavy metal ions can also be excluded as important candidates for induction of fibrosis and for elevated levels of the apoptotic index [46]. The concentration of heavy metal ions was extremely low. The level of sulphur was also less than 370 mg/kg dried alginate indicating the complete removal of fucoidan and other related sulphated mitogenic compounds [45]. Failure of heparinase treatment supports this view.

This finding of contamination of alginates with gram-positive bacterial spores and/or components rises the question how the algal material and/or the extracted alginate can be sterilised. Treatment of the algal material

with formaldehyde, glutardialdehyde or 70% ethanol is obviously not sufficient as indicated by the apoptosis assay. Antibiotics are obviously very efficient, but have the decisive drawback that they must be removed very carefully from the final product for medical application. Apart from this, a further problem is the water-tight demonstration of the absence of all spores in the final product which is not easy as shown by experiments going on in our laboratory at present. Sterilisation of the algal material by  $\gamma$ -irradiation, ethylene oxide or autoclaving are a priori the methods of choice, but leads to unpredictable reactions between the alginate, proteins and other components. Sterilisation of alginate powder or solutions is also not recommendable because it occurs at the expense of a dramatic reduction in viscosity. LV-alginates, however, do not allow the formation of long-term stable and functional transplants [5,10,12].

This demonstrates that the harvesting process and the quality of the algal material are the key features to overcome the problem of the production of sterile UHV-alginates. As shown by the above studies for bacterial contamination, high-quality stipes collected under benign conditions were free of such contaminations after purification. Future collection of appropriate material is obviously facilitated by the apoptosis assay because it yields the required information about impurities in time provided a laboratory is available. Additionally, it also allows routine validation of the individual steps of the extraction and purification process.

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