

Real-time 3-D dark-field microscopy for the validation of the cross-linking process of alginate microcapsules

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Abstract

Alginate-based microencapsulation is a promising method for long-term maintenance of cellular and membrane function of the cells and tissue fragments required for in vitro and in vivo biosensors, for tissue engineering and particularly for immunoisolation of non-autologous transplants. Microcapsules of high mechanical strength and optimum permeability can be produced by injection of BaCl₂ crystals into alginate droplets before they come into contact with external Ba²⁺. A key requirement is that the system parameters (number of crystals, speed of the crystal stream etc.) are properly adjusted according to the mannuronic and guluronic acid ratio and the average molecular mass of the alginate as well as to the diameter of the microcapsules. Robust, reliable, rapid and low-cost validation tools are, therefore, needed for assurance of the microcapsule quality. Here, we describe a novel three-dimensional (3-D) dark-field microscopy that allows the real-time measurement of the number and spatial distribution of the injected Ba²⁺ ions throughout the microcapsules after treatment with sulphate. This novel method requires only a conventional microscope equipped with three polarising filters and a double aperture stop. In contrast to confocal laser scanning microscopy images, peripherally attached BaSO₄ precipitates can clearly be distinguished from internal ones. The data also demonstrate that several steps of the alginate gelling process must be improved before such immunoisolation can be used in patients.

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1. Introduction

Immobilisation of cells in alginate matrices cross-linked with divalent cations allows cellular and mem-

brane functions to be maintained for much longer periods than for non-encapsulated cells. Potential applications of immobilised cells in agriculture, biotechnology and medicine are numerous. They include the production of chemicals and pharmaceuticals, selective and specific biosensors for long-term applications and use as the scaffold for tissue engineering [1–6]. The most exciting potential for immobilised cells entrapped in alginate gels is currently seen in the field of

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immunoisolated transplantation of allogeneic and xenogeneic cells [7–15]. Numerous animal studies [11,12,15] and pilot clinical trials with parathyroid tissue [9,12] have demonstrated the therapeutic efficacy of Ba^{2+} -alginate-encapsulated cell therapy. The recent introduction of highly purified alginate of extremely high viscosity was certainly an important step towards the clinical realisation of immunoisolated transplantation of non-autologous cells and tissue fragments into patients [12,15]. However, the long-term function of alginate/cell-based microcapsules and of in vivo biosensors relies upon the efficacy and reproducibility of the cross-linking process of the carboxyl groups of the polymeric mannuronic (M) and guluronic (G) acid chains. Conventional cross-linking (by dropping cell-loaded alginate into Ba^{2+} -containing isoosmolar solutions) generally produces microcapsules with poor homogeneity as diffusion limitations are considerable if the droplet diameter is greater than 600 μm [16]. Thus in vitro and in vivo stability is rather low as shown by NMR imaging and other methods [17,18]. Homogeneous cross-linking with external Ba^{2+} is possible if the cross-linking bath has a $\text{pH} < 5$ or if the incubation time at physiological pH is extended to several hours [17]. These conditions are, however, lethal for encapsulated cells. High cell viability and function can only be observed if the incubation in the 20 mM $BaCl_2$ solution is restricted to 15 min [12,15].

The recent introduction of the crystal gun method has solved the problem of inefficient and variable cross-linking of the alginate matrix [18]. Air pressure drives dried, sterilised $BaCl_2$ crystals or nanocrystals of $BaSO_4$ into the alginate droplets shortly before they come into contact with external Ba^{2+} . This efficient and simple method induces homogeneous cross-linking throughout the microcapsule, as demonstrated by confocal laser scanning microscopy (CLSM) and advanced $^1\text{H-NMR}$ imaging technology [17,18]. The viability and function of encapsulated cells is not affected by the crystal bombardment provided that the alginate concentration is adjusted properly. $^1\text{H-NMR}$ imaging, along with paramagnetic Cu^{2+} as contrast agent, and CLSM measurements have also given some evidence that the size of the droplet and the M/G ratio of the alginate determine the penetration depth of the crystals. Crystal gun parameters such as the density and speed of the $BaCl_2$ crystal stream must also be properly adjusted to obtain optimum cross-linking of the alginate droplet while simultaneously keeping the number of injected crystals within a non-lethal range. Appropriate information for optimisation and routine validation of microcapsule formation as required by granting agencies [19,20] can be obtained by NMR imaging and CLSM measurements, but these non-invasive evaluation techniques require highly sophisticated equipment that is not always available.

A solution to the problem is presented in this communication. The initial distribution of Ba^{2+} is determined by visualisation of solid $BaSO_4$ particles generated by immediate treatment of the microcapsules after gelling with 6 mM Na_2SO_4 saline solutions. The particles can be visualised by a novel and simple real-time three-dimensional (3-D) dark-field technique that provides a true microscopic 3-D image at magnifications between 25:1 and 400:1. This technique requires only a conventional microscope equipped with three polarising filters and a double aperture stop, thus meeting the demands for rapid, inexpensive and routine control of microcapsule quality. The potential of this technique is demonstrated here for microcapsules made of alginates exhibiting different M:G ratios and cross-linked either conventionally or by the crystal gun method.

2. Materials and methods

2.1. Alginates

For the production of ultra-high viscosity alginates of clinical grade, stipes of *Lessonia nigrescens* and *Lessonia trabeculata* (free of overgrowth by epiphytes and harvested freshly from the sea at Coquimbo, Chile) were used as starting material for extraction and purification of alginates. *L. nigrescens* grows in the tidal zone and is exposed to strong surf. The stipes are very elastic and flexible due to high-M alginate (about 60%; [21]). In contrast, *L. trabeculata* is found in depths between 0 and 30 m in subtidal habitats and is exposed to heavy currents. The stipes of this species are very stiff because of an extremely high content of G (about 90%; [22]).

Immediately after harvest, the outer (approximately 1–2 mm) layer was removed before the stipes were cut into small pieces. Then the pieces were subjected to antimicrobial treatment and dried outdoors under near sterile conditions for about 8 h. On arrival at Würzburg, Germany, the material was vacuum-sealed. Before extraction, the stipe pieces were physically cleaned to remove immunogenic surface proteins of non-plant origin that are frequently found in alginates after extraction and purification. Alginate was extracted using a mixture of appropriate Ca^{2+} -chelating agents and purified by repeated alcohol precipitation as described in detail elsewhere [15]. The viscosity of a 0.1% w/v purified alginate solution in distilled water was 20–40 mPa s. The complete removal of fibrosis-inducing impurities in the alginate was assessed by analytical assays as well as by the “modified mixed lymphocyte” and “apoptosis induction” assays [23–25]. Long-term animal studies (up to 1 year) have shown [26] that high-viscosity alginate of extremely high purity does not

evoke fibrosis and maintains function of encapsulated rat and human islets.

2.2. Microcapsule formation

For microcapsule formation, the purified alginates were dissolved in sterile 0.9% NaCl solution. The alginate concentration was adjusted to 0.65% w/v for the crystal gun method or 0.7% w/v for conventional cross-linking. In some experiments 3% human serum albumin (HSA; Octapharma, Langenfeld, Germany) were additionally added to the alginate solution because of the beneficial effect of HSA on microcapsule stability and on cell viability and function [27].

Microcapsules of about 600 μm diameter were produced with the two-channel air-jet droplet generator as described in Ref. [15]. Cross-linking of the droplets by external Ba²⁺ was performed in a 20 mM BaCl₂ solution adjusted to 290 mOsm using NaCl buffered at pH 7.0 by 5 mM histidine. The crystal gun arrangement used for internal gelling is described in detail in Ref. [18]. The very hygroscopic BaCl₂ crystals were heated at a temperature of 150 °C for 2 h before injection in order to remove any water. All gelling of alginate droplets was performed under sterile conditions and the solutions and the crystal air jet were sterilised before use.

If not stated otherwise, microcapsules were removed from the BaCl₂ solution after 15 min. To obtain information about the initial 3-D Ba²⁺-distribution within the microcapsules, excess (free) Ba²⁺ ions were precipitated as BaSO₄ by treatment with an isoosmolar 6 mM Na₂SO₄ saline solution for 30 min. For microscopy the sulphate-treated microcapsules were incubated in 0.9% NaCl solution.

2.3. Real-time 3-D dark-field microscopy

We modified the well-established technique of real-time single lens 3-D microscopy (originally developed for high resolution bright-field, Nomarski or phase contrast optics; [28,29]) to give information about the number and spatial distribution of internal BaSO₄ precipitates. This technique is based on oblique transmitted-light illumination. The right and left images are separated by polarising or red/green anaglyphic filters, which serve as an image switch, allowing real-time 3-D microscopy with high resolution. Unmodified, this technique does not work for dark-field illumination because the scattered light emitted from the microscopic object is no longer polarised. Therefore, the first part of the image switch must be shifted to the rear focal plane of the objective lens (Fig. 1(A)), although this causes an unavoidable loss of lateral resolution. Two circular apertures arranged closely side by side and each 1/3 of the diameter of the back focal plane of the objective give the best compromise between depth-of-field, perceived

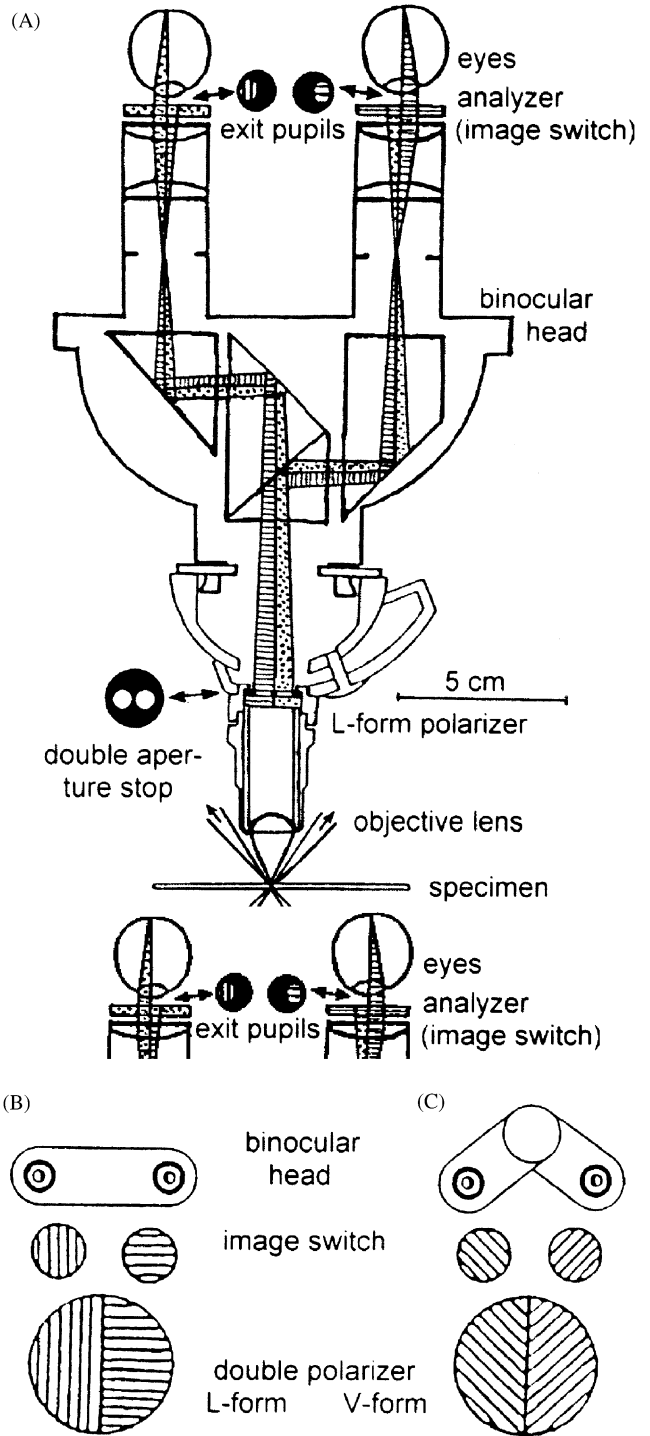


Fig. 1. Principle of real-time 3-D dark-field microscopy technique developed for rapid visualisation of the spatial distribution of BaSO₄ precipitates within alginate microcapsules. (A) Optical path with polarising filters serving as image switches; (B, C) alternative binocular heads together with the corresponding L- or V-form double polarising filters. For more details, see text.

spatial depth and resolution while minimising distortions of image detail (see Fig. 1(A)). For classical transmitted-light dark-field illumination at medium magnifications (objective lenses between 2.5:1 and

25:1), ring-shaped apertures as designed for phase contrast microscopy (“Ph2” or “Ph3”) can be used. Loss of lateral resolution can be compensated by using high aperture apochromatic or fluorite objective lenses (e.g. Fluotar 10/0.4 Wild, Heerbrugg, Switzerland).

The resulting two light beams are then polarised (mutually perpendicular) by an L-form (or V-form, depending on the binocular head; see Figs. 1(B) and (C)) double polarising filter mounted close to the apertures. There is also a polariser in each eyepiece, so each eye receives the image formed through the corresponding half of the objective lens.

For 3-D imaging the filters in the eyepieces must be carefully adjusted. As depicted in Figs. 1(B) and (C), images of the apertures are formed some 10–15 mm above the eyepieces. To obtain proper spatial depth information, each eyepiece and its polarising filter must be rotated until it images only the corresponding aperture. If both eyepieces are rotated by 90°, the left and right images are interchanged and the microcapsule is seen spatially inverted.

2.4. Confocal laser scanning microscopy

As a control, BaSO₄ precipitates of sulphate-treated microcapsules were also investigated in the reflection mode (i.e. with dark-field epi-illumination) at 488 nm line of an air-cooled argon laser of a CLSM microscope (Leica TCS NT, Bensheim, Germany) as described previously [18]. Under these conditions the BaSO₄ precipitates appear as bright spots clearly distinguishable from the background. For image acquisition, the microcapsules were placed in a flat plastic tube filled with saline solution. The images were processed using Amira (TGS, San Diego, California, USA) and Photoshop 6.0 (Adobe Systems Inc., California, USA).

3. Results

3.1. Confocal laser scanning microscopy

CLSM images of Ba²⁺ microcapsules made of high-M alginate extracted from *L. nigrescens* confirmed previous results [18]. Inspection of appropriate optical sections demonstrated (data not shown) that the number of BaSO₄ precipitates located within the microcapsules increased dramatically when the crystal gun method was used instead of the conventional method for cross-linking. As expected, due to the spherical nature of the microcapsule and the shape of the BaCl₂ crystal stream, the number of precipitates varies throughout the optical sections. Thus, for an unambiguous and quantitative determination of the number of internal precipitates a 3-D reconstruction is required. Screening experiments showed that at least 50 sections are necessary to meet the

demands of an unambiguous quantisation of the precipitates throughout the interior of the microcapsules. A typical 3-D pair reconstructed from 64 optical sections made on an internally gelled microcapsule is shown in Fig. 2(A). It is obvious that the spatial distribution and number of internal BaSO₄ precipitates cannot accurately be determined, particularly in the cap regions because of the many precipitates adhering to the outer surface of the microcapsule. Both peripheral and internal precipitates are in focus and cannot, therefore, be distinguished by the human eye. Due to this problem, 3-D pairs can only be evaluated provided that the caps of the microcapsule are omitted (Fig. 2(B)). This has, however, the disadvantage that the spatial distribution of the precipitates cannot quantitatively be determined throughout the entire microcapsule.

With the high-G alginate extracted from *L. trabeculata* similar results were obtained, except that the absolute number of precipitates was much less in

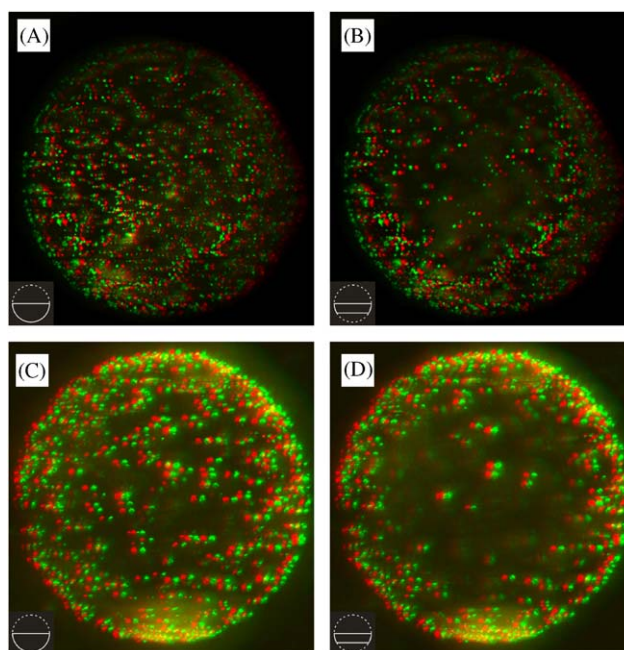


Fig. 2. Anaglyphic 3-D images of a hemisphere of a microcapsule visualised by confocal laser scanning microscopy (CLSM; Leica TCS NT, Bensheim, Germany). The approximate focal plane in which the scanning was performed is given on the left lower side of each image. The microcapsules were made up of alginate extracted from *L. nigrescens* (high-M; 0.65% w/v; A, B) and *L. trabeculata* (high-G; 0.65% w/v; C, D). Ba²⁺-cross-linking was achieved by the crystal gun method. Microcapsules were treated with sulphate immediately after the cross-linking process. (A and C): 3-D images of microcapsule hemispheres (viewed from the centre to the periphery; reconstructed from 64 and 128, respectively, optical sections). (B and D): 3-D images of a central part of the same microcapsules as in (A) and (C) reconstructed from 32 and 64, respectively, optical sections (see insets). Comparison of corresponding images demonstrates that internal precipitates can only be distinguished clearly from peripheral ones if the cap is completely removed. For viewing of the anaglyphic 3-D images red/green or red/blue glasses or filters must be used.

internally gelled microcapsules (see Figs. 2(C) and (D)). As in the case of high-M alginate, quantitative evaluation of the spatial distribution of internal BaSO₄ precipitates was only possible after omission of the caps (Fig. 2(D)).

However, because of the time-consuming procedure and the limited access to CLSM equipment it was not possible to investigate more than a few microcapsules. Statistically weighted conclusions were, therefore, not possible in reasonable time.

3.2. 3-D dark-field microscopy

The 3-D dark-field technique does not share these disadvantages. In contrast to CLSM, a quantitative evaluation of the spatial distribution of BaSO₄ precipitates throughout the interior of the microcapsules was possible due to the inherent limited depth-of-focus even with many precipitates adhering to the outer surface. The number and distribution of internal precipitates could easily and rapidly be determined by continuously focussing through the microcapsules (Fig. 3(A)). Consistent with the CLSM analyses, application of the crystal gun technique increased the number of internal precipitates (Figs. 3(B)–(D)). The number of precipitates greatly increased both for high-M and high-G alginates and 1:1 mixtures when the crystal gun was used for cross-linking (Figs. 3(B) and (C)). Microcapsules made in the conventional way contained 6.7 ± 3.8 (high-G), 12.2 ± 5.8 (high-M) and 9.8 ± 5.2 (1:1 mixture of high-M and high-G) precipitates (mean value \pm SD of three independent experiments). As indicated by these average values, the number of precipitates varied considerably. The variation arose partly from the size of the microcapsules, as the number of precipitates increased with the size of the microcapsules. Interestingly, some microcapsules had an asymmetric distribution of peripheral precipitates with more in one hemisphere than the other (data not shown, but see Fig. 3(D)). In addition, evaluation of many 3-D images showed that the size of the internal precipitates was not always uniform and that the precipitates were not evenly distributed. Such inhomogeneities may affect cross-linking and long-term microcapsule stability.

The 3-D dark-field technique allowed rapid optimisation of the BaCl₂-injection parameters and thus of the cross-linking process. For example, removal of very small and very large crystals before use and concomitant reduction of the speed of the crystal jet resulted in a rather homogeneous spatial distribution of fairly evenly sized precipitates within the microcapsule (data not shown). Equally, the 3-D dark-field technique also demonstrated that the standard washing process for microcapsules intended for transplantation was suboptimal. For this application, the microcapsules were washed three times with 0.9% NaCl solution before

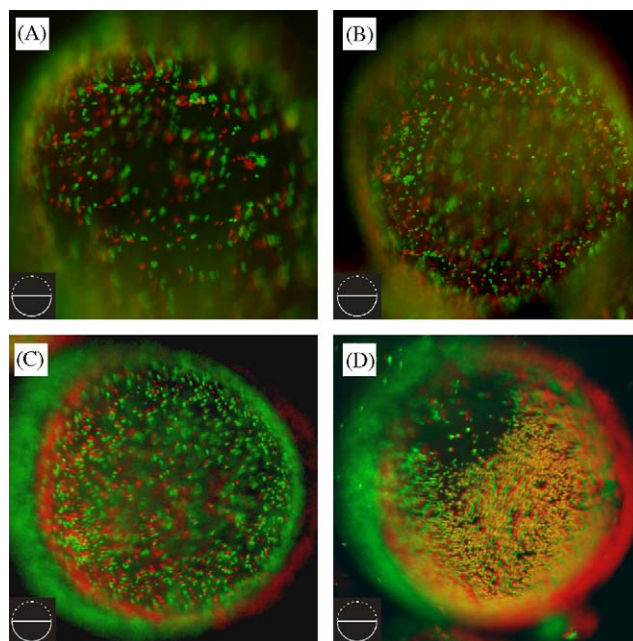


Fig. 3. 3-D dark-field images of alginate microcapsules visualised by the microscope shown in Fig. 1. As in Fig. 2 microcapsules were treated with sulphate immediately after the cross-linking process. (A and B): Alginate extracted from *L. nigrescens* (high-M; 0.65% w/v) and cross-linked conventionally (A) and with the crystal gun method (B). (C) BaSO₄ precipitates within a microcapsule made up of a 1:1 mixture of *L. nigrescens* and *L. trabeculata* alginate (0.65% w/v) and cross-linked with the crystal gun method. (D) Asymmetric distribution of BaSO₄ precipitates (above left) cross-linked with the crystal gun method (1:1 mixture of alginate extracted from 0.65% w/v *L. nigrescens* and *L. trabeculata*). For viewing of the anaglyphic 3-D images red/green or red/blue glasses or filters must be used. Under these conditions it is obvious that the internal precipitates are in focus, whereas the precipitates in the periphery and the aqueous microsurrounding of the microcapsules are out of focus due to the inherent limited depth-of-focus of the 3-D dark-field image technique. Thus, the distribution and number of internal precipitates can be clearly distinguished from the outer ones without removal of the microcapsules caps as required for the CLSM technique (see Fig. 2).

excess BaCl₂ was removed by precipitation with Na₂SO₄. The 3-D images of microcapsules treated in this manner showed BaSO₄ still adhering to the outer surface after this procedure (data not shown). These precipitates may serve as nuclei for adsorption of proteins from body fluids. It is well known [12] that this can lead to cell attachment, migration and release of cellular material resulting ultimately in fibrotic overgrowth of the microcapsule. 3-D dark-field images of microcapsules treated with different washing regimes showed that smooth surfaces can be obtained when the three NaCl washing steps and the subsequent sulphate treatment are followed by vigorous stirring of the microcapsules for up to two days in culture medium. Under these conditions the attached precipitates were completely removed from the outer microcapsule surface.

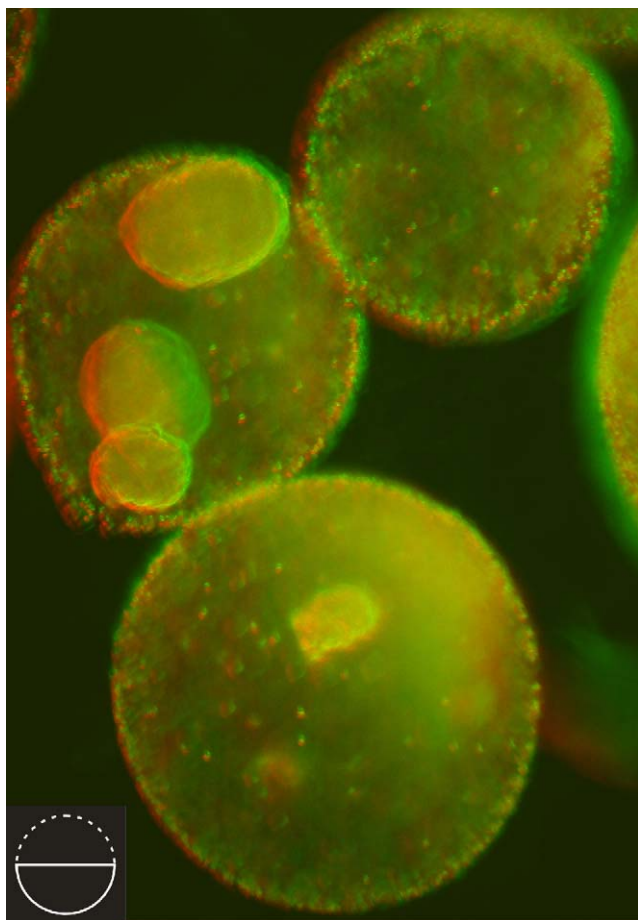


Fig. 4. 3-D dark-field image of a microcapsule containing rat islets. The microcapsule was made of alginate extracted from *L. nigrescens* and *L. trabeculata* (0.65% w/v alginate, 1:1 mixture). For viewing of the anaglyphic 3-D images red/green or red/blue glasses or filters must be used. Note that the number of internal precipitates is less because of the large volume occupied by the islets.

3-D dark-field technique is also a useful tool for optimisation of encapsulation of cells and tissues. An example is given in Fig. 4. It is obvious that BaSO_4 precipitates can clearly be resolved in the region surrounding entrapped Langerhans' islets.

4. Discussion

The above data demonstrate that the novel 3-D dark-field microscopy introduced here is superior to CLSM for statistical evaluation and routine validation of the efficacy of the cross-linking process of alginate droplets as needed for immunoisolated transplantation, tissue engineering, controlled drug release from immobilised cells and cryopreservation of immobilised cells as well as for the development of biosensors based on cells [3,4,13,30]. Drawbacks of the CLSM technique are the limited access to this highly sophisticated, expensive equipment in many laboratories and the extremely time-

consuming process of a 3-D reconstruction of a single microcapsule, since many optical sections are required. The extended focus of CLSM imaging obviously represents a further and rather pivotal disadvantage. All precipitates within a given optical section are in focus and appear rather similar. Therefore, it is not possible for the human eye to unambiguously discriminate between precipitates located inside a microcapsule from those adsorbed outside. The spatial distribution of precipitates within the microcapsule can only be quantified after removal of the cap regions (see Figs. 2(B) and (D)). In consequence, valuable information about the spatial distribution of the precipitates within the cap regions is lost.

In contrast to CLSM, the field depth of 3-D dark-field microscopy is restricted. Thus, precipitates at the periphery can easily be distinguished from those in the interior of the microcapsule. Provided that the microscope is additionally equipped with three polarising (or anaglyphic) filters and appropriate double aperture stops (see Fig. 1), real-time 3-D information about the spatial distribution of the precipitates throughout the entire microcapsules can easily and quantitatively be obtained by continuously focussing from the upper to the lower cap. A great advantage of this technique is that, without dismounting the lenses, the two polarising filters and the two aperture stops can be installed in all microscopic objectives with their focal plane situated close to or even outside of the rear lens. If higher magnifications are needed (e.g. in the case of tiny alginate microcapsules or thin sheets as required e.g. for controlled drug release), the polarising filters as well as the aperture stops must be mounted inside the objective, i.e. at the level of the rear focus plane of the objective. This can easily be done by a lens manufacturer.

The 3-D dark-field technique described here has some minor disadvantages. Due to the distance between the aperture stops the spatial depth in z -direction is somewhat exaggerated and thus the spherical microcapsules appear of ovoid shape. Furthermore, due to the out-of-focus images of the crystals, the core of the microcapsules can appear fuzzy. This effect increases with the number and size of the precipitates localised at the periphery of the upper cap. This is obviously not a problem for routine evaluation of microcapsules for animal and clinical applications because in this case, BaSO_4 precipitates must be completely removed from the surface in order to avoid surface roughness. As shown above the success of Ba^{2+} treatment can easily be controlled by the 3-D dark-field technique.

The measurements reported here have also demonstrated that the penetration depth of the BaCl_2 crystals depended on the M:G ratio of the alginate. High-G alginate extracted from *L. trabeculata* had fewer internal precipitates than high-M alginates extracted from *L. nigrescens* or 1:1 mixtures of high-M and high-G

alginate. In the light of the egg-box model [2] this finding suggests that G–G alginate blocks in the peripheral layer of the microcapsule become more tightly cross-linked when coming in contact with the BaCl₂ crystals than alginate M–M blocks, thus preventing further penetration of crystals. This conclusion is also supported by reduced insulin release from islets encapsulated in high-G alginate as opposed to those encapsulated in high-M alginate (unpublished data). Interestingly, microcapsules made of 1:1 mixtures of high-M and high-G alginate have yielded similar results as high-M alginate microcapsules. The number and spatial distribution of precipitates were comparable with those in high-M alginate microcapsules, but the biocompatibility of the mixed alginate was extremely high. 4-week transplantation studies in diabetic mice did not give any evidence for fibrotic overgrowth (unpublished data). Long-term in vitro studies have also shown that microcapsules made of mixed alginate are extremely stable (manuscript in preparation). Thus, in the light of these data and the results presented here mixtures of *L. trabeculata* and *L. nigrescens* alginates seem to be the favoured scaffold for encapsulation of islets and other cells as well as for the manufacture of cell-based biosensors.

One puzzling problem remains, i.e. why precipitates attached to the outer surface are often seen predominantly in one hemisphere of the microcapsule (see e.g. Fig. 3(D)). Speculation that this hemisphere was oriented towards the BaCl₂ crystal stream does not explain the asymmetric distribution of precipitates in microcapsules made in the conventional way. Therefore, it is more likely that this finding can be taken as evidence that deformation of the alginate droplets and/or dragging of air during entry into the BaCl₂ solution prevents one hemisphere from coming into immediate contact with the cross-linking agent. Such processes could also be responsible for the finding that islets (and other cells) are not always centred within the microcapsule after the end of the cross-linking process (see Fig. 4).

Evaluation of the alginate dropping process by use of high-speed cameras together with 3-D dark-field microscopy will certainly help to elucidate the liquid-contact processes involved and to launch the strategy of cell-encapsulated therapy for clinical trials.

5. Conclusions

Real-time 3-D dark-field microscopy is a rapid and cheap tool for validation of the cross-linking process of alginate microcapsules. Aliquots of cells-containing microcapsules prepared for immunoisolated transplantation, tissue engineering or use in biosensors are treated with sodium sulphate and the distribution and number

of BaSO₄ precipitates can be easily determined by continuous focussing throughout the microcapsules incubated in culture medium. This yields information about the quality of the cross-linking process of the polymeric chains of the alginate as required by granting agencies for medical applications.

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