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E.B. Hunziker · J. Wagner · D. Studer

Vitrified articular cartilage reveals novel ultra-structural features respecting extracellular matrix architecture

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Abstract The quality of cryosections prepared from high pressure frozen bovine articular cartilage has been recently evaluated by systematic electron diffraction analysis, and vitrification found to be zone-dependent. The lower radial layer was optimally frozen throughout the entire section thickness (150 μm), whereas in the upper radial, transitional and superficial layers this was achieved down to a depth of only approximately 5–50 μm. These differences were found to correlate proportionally with proteoglycan concentration and inversely with water content. In the current investigation, extracellular matrix ultrastructure was examined in high pressure frozen material (derived from the lower radial zone of young adult bovine articular cartilage), by both cryoelectron microscopy of cryosections and by conventional transmission electron microscopy of freeze-substituted and embedded samples. Several novel features were revealed, in particular, the existence of a fine filamentous network; this consisted of elements 10–15 nm in diameter and with a regular cross-banded structure similar to that characterising collagen fibrils. These filaments were encountered throughout the entire extracellular space, even within the pericellular region, which is generally believed to be free of filamentous or fibrillar components. The proteoglycan-rich interfibrillar/filamentous space manifested a fine granular appearance, there being no evidence of the reticular network previously seen in suboptimally frozen material.

Introduction

Articular cartilage has a principally biomechanical function, the structural correlate of which resides in its intercellular substance. The extracellular matrix, which consists of proteoglycans, fibrillar collagens and a number of other proteins, constitutes approximately 90% of the total tissue volume at maturity; the remaining 10% is represented by chondrocytes, which are responsible for its synthesis, degradation and maintenance (Freeman 1973; Stockwell 1979). A characteristic trait of articular cartilage is its anisotropic organisation into five morphologically distinct layers, which are also distinguishable on the basis of molecular criteria; these are designated as the superficial, transitional, upper- and lower radial, and calcified zones. In each layer, the intercellular substance is divided into clearly delineated pericellular, territorial and interterritorial matrix compartments on the basis of differences in fibrillar collagen architecture (Eggli et al. 1985; Freeman 1973; Stockwell 1979). In the proteoglycan-rich pericellular (lacunar) compartment, fibrillar collagens are generally believed to be absent, whereas in the territorial matrix they form a basket-like mantle around chondrocytes and chondrones (groups of chondrocytes). These two compartments together constitute only about 5% of the total matrix volume, the bulk proportion (95%) being occupied by the interterritorial substance, within which collagen fibrils run predominantly in parallel. The general orientation and thickness of fibrils are, however, zone-specific (Ghadially 1983; Weiss et al. 1968), as is the concentration of the interfibrillar proteoglycan molecule population, which is lowest in the superficial and tangential zones, and highest in the lower radial one (Maroudas et al. 1969; Studer et al. 1995).

These current concepts concerning matrix organisation are to a large extent based upon data gleaned from morphological analysis of chemically preserved tissue. It is well known that biological material processed by these means is subject to many structural disturbances (such as shrinkage phenomena), engendered by aqueous shifting and extraction of macromolecules, which limit resolution (Engfeldt and Hjertquist 1968; Hunziker und Graber 1986).

Processing of tissue by cryotechnical means represents an alternative approach, which circumvents many of the problems giving rise to artefacts under conventional chemical fixation conditions and has the potential to yield a state of preservation closely resembling the native one

E.B. Hunziker (✉) · J. Wagner · D. Studer
M. E. Müller Institute for Biomechanics, University of Bern, Murtenstrasse 35, PO Box 30, CH-3010 Bern, Switzerland
Tel. +41 31 632 86 86; Fax +41 31 632 49 55

(Sitte et al. 1987; Studer et al. 1989). Previous reports have shown the lateral resolution limits for ultrastructural analysis of cartilage matrix to be considerably improved by such means (Keene and McDonald 1993). This potential may be realized only if tissue is preserved in a vitrified state, viz., freezing conditions need to be such that ice crystal formation and growth are completely avoided. The only methodology available for achieving vitrification of bulk biological specimens is high pressure freezing (Moor 1987; Muller and Moor 1984). Recent theoretical and experimental data concerning this technology (Engfeldt et al. 1994; Hunziker 1993) have revealed the previously claimed vitrification depth of 600 μm for tissue slabs to be much too optimistic. Such estimates were based upon morphological inspection of freeze-etched and freeze-substituted specimens, in which the absence of segregation patterns served as the criterion for judging whether vitrification had occurred (Dahl and Staehelin 1989; Hunziker and Schenk 1984; Hunziker et al. 1984; Muller and Moor 1984; Studer et al. 1989).

Electron diffraction analysis of ultrathin cryosections affords a means of ascertaining whether vitrification has been effected in biological specimens (Dubochet et al. 1988). In the current study, these tools (cryoultramicrotomy and electron diffraction analysis), in combination with improved high pressure freezing techniques, were implemented to reassess the ultrastructure of young adult bovine articular cartilage extracellular matrix.

Materials and methods

High pressure freezing

Humeral head articular cartilage (central region) was derived from the shoulders of young adult (10–14 months) cows (Red-Holstein crossbred with Brown Swiss and Simmenthaler strains) obtained from a local abattoir; these joints were maintained at 277 K and processed within 12–14 h of death. Material from a total number of eight animals (approximately 3–5 samples being derived from each cow) was processed. In order to confirm information supplied with regard to freshness, cell viability was assessed in cultured tissue slices obtained from the first few animals by monitoring [^{35}S]sulphate incorporation into chondrocytes; autoradiographic analysis revealed 100% survival (data not presented). Cylinders comprised of the full articular cartilage thickness, plus a layer of subchondral bone, were produced using a 4-mm-diameter myelotomy drill (Straumann Institute, Waldenburg, Switzerland). The subchondral bone was removed and discarded, the remaining cartilage cylinder being dissected longitudinally into two halves. One half-cylinder was glued with its longitudinally cut flat side onto the specimen holder of an OTS-3000-03 vibratome (EMS, USA) using a rapidly bonding adhesive (Aron Alpha, Borden, USA), and tissue slices, 150 μm in thickness, were cut perpendicular to the joint surface in a bath of 1-hexadecene (Fluka, Buchs, Switzerland). From these slices, discs, 1.7 mm in diameter, were then punched out using a precision stainless steel hole punch (Grieshaber, Schaffhausen, Switzerland). These were positioned within a custom-built specimen holder, which consisted of a steel spacer ring (inner diameter 2 mm, outer diameter 3 mm, depth corresponding to tissue slice thickness, i.e., 0.15 mm), sandwiched between two cylindrical aluminium plates (diameter 3 mm, thickness 0.2 mm). To compensate for the remaining space between the sample sandwich and the holder cavity, an additional spacer ring was introduced. Possible spaces remaining between sample and plates (engendered by tissue surface irregularities) were filled with 1-hexadecene (Studer et al. 1989).

The specimen sandwich was loaded into a special sample holder and frozen under high pressure (2045 bar) in a Leica EM HPF high pressure freezer (Leica, Vienna, Austria). A chromel-alumel thermocouple was used to record the temperature and a piezoelectric sensor (Burster, Präzisionswerkzeuge Gernsbach, Germany) to monitor the pressure curve. Prior to each experiment, temperature and pressure profiles were simultaneously recorded using an oscilloscope and printer (Hameg, Frankfurt, Germany).

After removal from the apparatus, the frozen specimen sandwiches were immediately transferred to liquid nitrogen for storage at atmospheric pressure. High pressure frozen material was destined either for cryosectioning with subsequent cryoelectron microscopy and electron diffraction analysis, or freeze-substitution followed by conventional embedding in epoxy resin and ultrastructural analysis in the transmission electron microscope.

Cryosectioning with subsequent cryoelectron microscopy and electron diffraction analysis

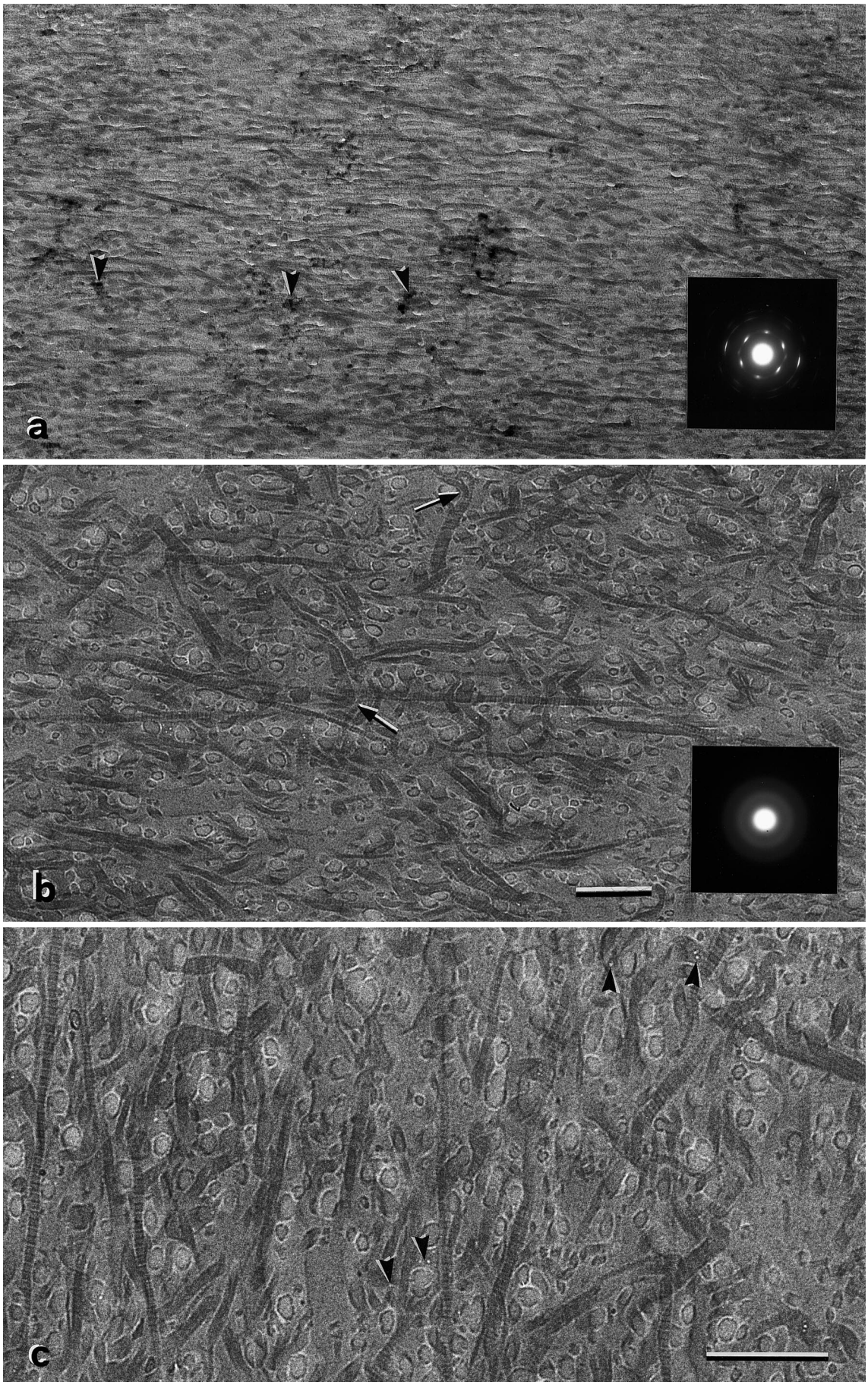
High pressure-frozen cartilage discs were removed from the metal sandwich within the FCS-cryochamber of a Reichert Ultracut S microtome, cooled to 105 K and clamped in a custom-built holder as previously described (Michel et al. 1991). The frozen cartilage tissue was finely trimmed and cryosectioned using a 30° angle diamond knife (Diatome, Biel, Switzerland). Cryosections were transferred to 400-mesh carbon-coated copper grids (Michel et al. 1992) and immersed in liquid nitrogen until required for use.

Cryosections maintained at 100 K were examined at an acceleration voltage of 100 kV in a Hitachi H-7100 transmission electron microscope using a Gatan cryoholder (Gatan, Warrendale, Pa, USA). Micrographs were taken at a primary magnification of ×10000 (estimated maximum electron dose 1500–3000 e/nm^2, prior to and including the first exposure). Imaging and focusing were carried out on the same section area. The latter was achieved with the help of the wobbler (no visible movement), and defocusing set at 3–5 μm for optimisation of contrast. Electron diffraction was performed using a camera length of 0.4 m. Images were recorded on Agfa Scientia 23D 56 plates. Brightness was set in order to achieve exposure times of 2–6 s (automatic imaging mode). In the absence of vitrification, the presence of (hexagonal) ice crystals within the tissue produces Bragg reflections and an electron diffraction pattern characterised by clearly defined spots (Fig. 1a); these are absent in truly vitrified (amorphous) tissue (Fig. 1b).

Freeze-substitution followed by conventional embedding in epoxy resin and ultrastructural analysis in the transmission electron microscope

High pressure-frozen cartilage discs were removed from the metal sandwich whilst immersed in liquid nitrogen and freeze-substitu-

Fig. 1a–c Ultrathin cryosections of high pressure-frozen mature bovine articular cartilage. **a** Tissue water has been frozen in a crystalline condition, as revealed by its electron diffraction pattern (*insert*), which exhibits clearly defined peripheral spots. A fine segregation pattern is detectable within the interfibrillar matrix, and the occurrence of Bragg reflections (*arrowheads* in **a**, indicating the presence of crystals) is associated with tissue frozen in this condition. **b, c** Tissue water has been truly vitrified, as revealed by the diffuse electron diffraction pattern (*insert* in **b**). Some fibrils exhibit abrupt changes in their course (*arrows* in **b**). Cross-sectioned collagen fibrils manifest a distinct staining pattern, characterised by a pale central body and a darker peripheral ring; profiles are, in turn, surrounded by a light perifibrillar halo. Electron bombardment causes bubbling of structures rich in organic material (white spots; *arrowheads* in **c**). *Bars*, 500 nm


a
b
c

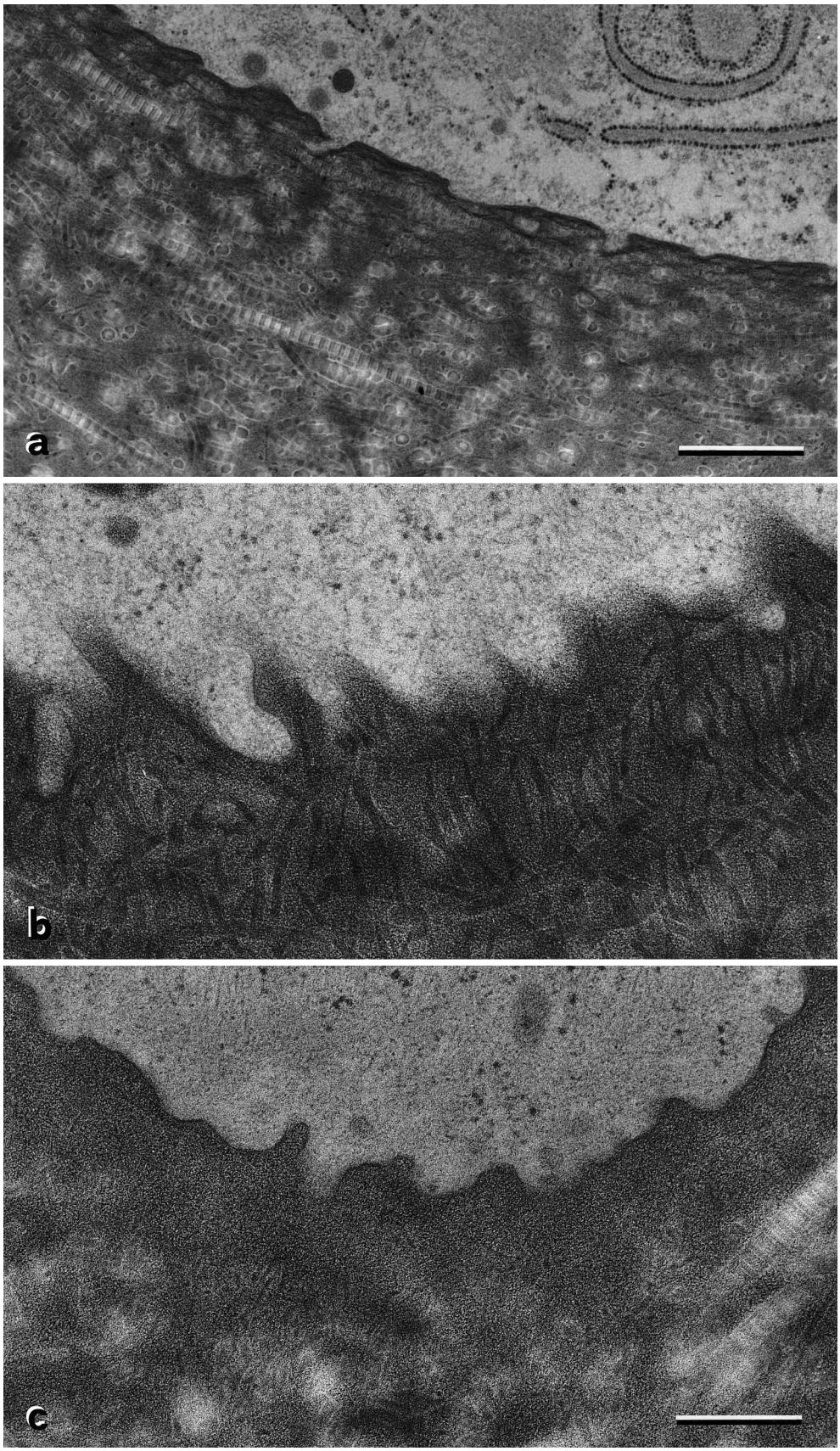


Fig. 2a–c Electron micrographs illustrating pericellular regions in high pressure-frozen and freeze-substituted/embedded bovine articular cartilage tissue. The fibrillar/filamentous composition of the pericellular matrix is seen to vary considerably. **a** Fibrils and filaments are encountered in the immediate vicinity of the chondrocyte periphery. **b** Only filaments are found in this zone. **c** This region contains neither filaments nor fibrils. *Bars* **a** 800 nm, **b, c** 400 nm

tion then carried out in a liquid nitrogen-cooled cryostat. Frozen tissue was substituted by anhydrous acetone, in both the absence and presence of 2% osmium tetroxide (Steinbrecht and Muller 1987), in three stages; 17 h at 183 K, 12 h at 213 K and 12 h at 243 K. The specimens were then maintained at a temperature of 273 K for 1 h, washed 3 times in anhydrous acetone and embedded stepwise in Epon 812 (30%, 70%, 100% resin). The infiltration times were 3 h for the first two embedding steps (30% and 70%) and 3 days in pure resin. Polymerisation (in fresh resin) was carried out at 333 K for 5 days.

Ultrathin sections of plastic-embedded specimens were cut on a Reichert Ultracut E microtome equipped with a 45° angle diamond knife (Diatome). Ultrathin sections (60–80 nm) were stained for 7 min with uranyl acetate and lead citrate in preparation for examination in a Hitachi H-7100 transmission electron microscope using an acceleration voltage of 75 kV.

Results

Novel features relating to extracellular matrix ultrastructure in young adult bovine articular cartilage tissue preserved by high pressure freezing

In articular cartilage matrix, which is known, on the basis of electron diffraction analysis, to be truly vitrified (Fig. 1b, c), novel ultrastructural features relating to collagen fibrils and the proteoglycan-rich interfibrillar space were observed.

In *cryosections*, longitudinally sectioned collagen fibrils disclosed a regular cross-banding with a periodicity of approximately 67 nm (and a subperiodicity of approximately 22.5 nm). In a number of instances, they underwent abrupt changes in their course (Fig. 1b). Cross-sectioned collagen fibrils showed a pale body and a dark peripheral ring (Fig. 1b, c). In *freeze-substituted/embedded tissue*, these differences in contrast were likewise mirrored in the staining pattern. Fibrils were surrounded by an electron-lucent perifibrillar halo (Figs. 1b, c, 3, 4a), which appears to represent a distinct water-rich layer surrounding collagen II fibrils (Studer et al. 1996). Fibrillar collagen was often, but not consistently, observed within the pericellular matrix compartment (Fig. 2a).

Interspersed between the collagen fibrils of *freeze-substituted/embedded tissue*, was a network of fine cross-banded filaments, 10–15 nm in diameter (Figs. 2a, b, 3). This network was not confined to one matrix compartment but was distributed throughout the entire extracellular space, albeit at differing densities; hence, it was also encountered within the pericellular matrix compartment, sometimes in conjunction with collagen fibrils (Fig. 2a), sometimes alone (Fig. 2b). Occasionally, neither filaments nor collagen fibrils were observed here (Fig. 2c). The periodic banding of these filaments was similar to that manifested in collagen fibrils, which suggests that they are collagenous in nature. Indeed, a perifilamentous electron-lucent halo was also seen in association with these structures (Fig. 3).

In vitrified *cryosections*, proteoglycan-rich interfibrillar (filamentous) spaces displayed an evenly distributed, fine granularity (Fig. 1b, c), which contrasted markedly with the clumped (or reticular) appearance evinced in suboptimally frozen (crystalline) tissue (Fig. 1a). In *cryosectioned material*, a distinction between vitrified and crystalline extracellular matrix can be made quite inde-

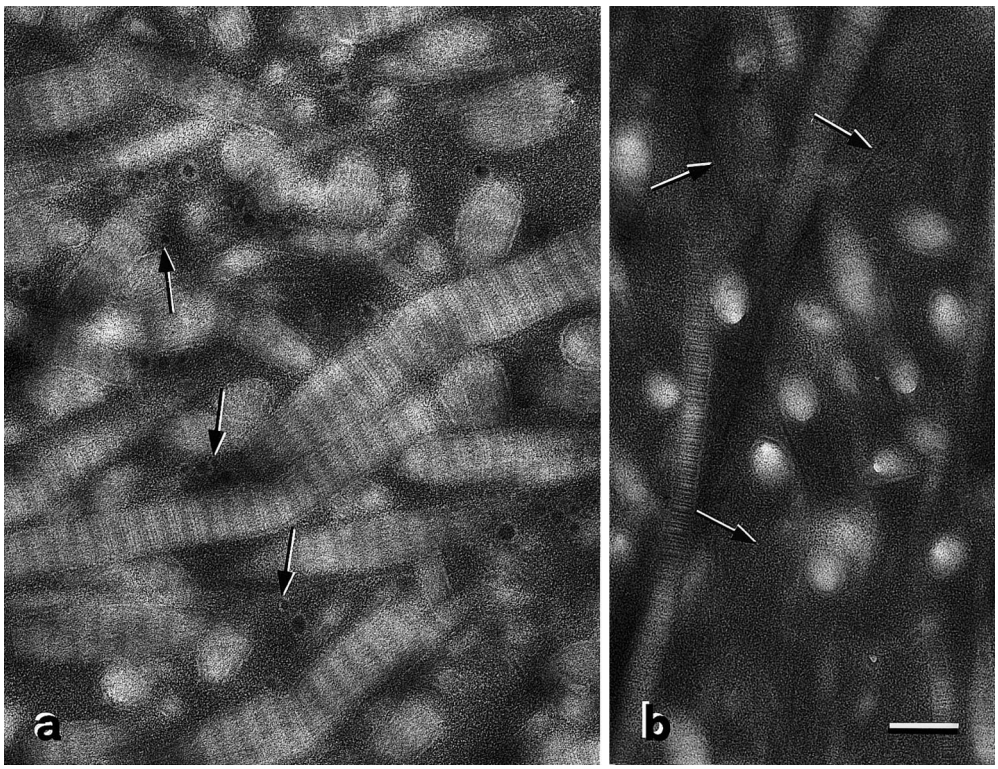


Fig. 3a, b High magnification electron micrographs of mature bovine articular cartilage matrix (lower radial zone) after high pressure freezing, freeze-substitution and Epon-embedding. A network of fine cross- (**a**) and longitudinally sectioned (**b**) filaments (10–15 nm in diameter; see *arrows*) is apparent between collagen fibrils, which exhibit a periodicity in the order of 67 nm. *Bar*, 200 nm

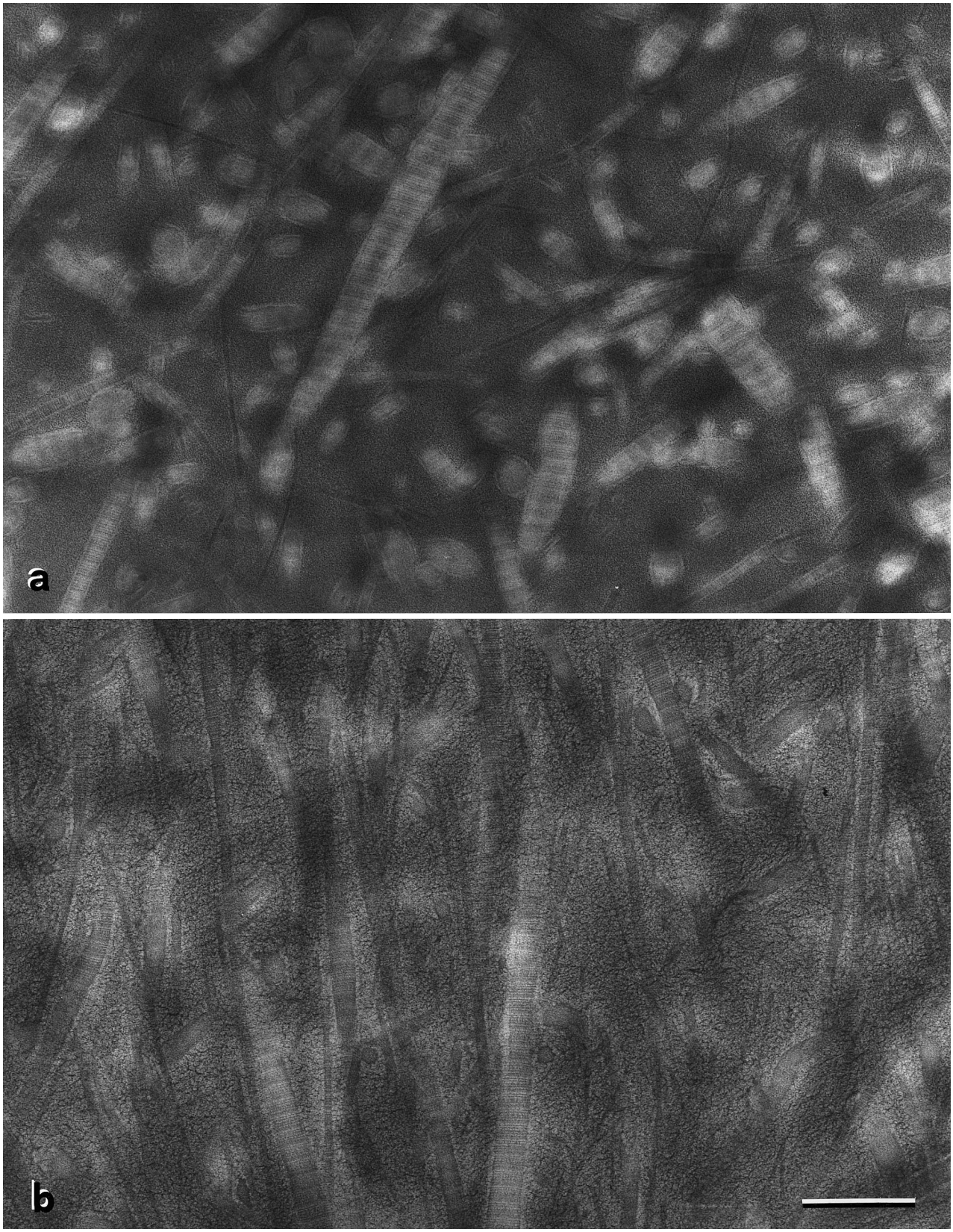


Fig. 4 Electron micrographs of mature bovine articular cartilage after high pressure freezing, freeze-substitution and Epon-embedding, illustrating optimally (**a**) and suboptimally (**b**) frozen tissue. In **a**, the ground substance interspersed between filaments and collagen fibrils is seen to have a fine granular appearance, whereas in **b** it is revealed as a coarse reticular network. Bar, 500 nm

pendently of morphological indicators, i.e., on the basis of tissue section origin, since systematic electron diffraction analysis (Studer et al. 1995) has shown that the lower radial zone is vitrified across the entire thickness (150 μm) of a high pressure-frozen specimen, whereas the upper radial, transitional and superficial layers are optimally frozen down to a depth of only 5–50 μm. As in cryosections, the proteoglycan-rich interfibrillar spaces of vitrified, *freeze-substituted/embedded tissue* also exhibited an evenly-distributed, fine granularity (Fig. 4a); the reticular network previously described by ourselves (Hunziker et al. 1984) and others (Arsenault and Kohler 1994; Engfeldt et al. 1994) was not observed. Indeed, this latter manifestation is now recognised to be a segregation pattern elicited by ice crystal-induced phase separation, since it was seen exclusively in suboptimally frozen areas of the superficial and transitional zones (Fig. 4b).

Discussion

Cryoprocessing techniques hold the potential for preserving tissue structures in a near-native condition, but this can be realised only if water is frozen in its liquid state, i.e., if true vitrification is achieved. When ice crystals begin to form and grow, tissue components become segregated into phases of pure water (ice) and of concentrated biological residues (organic materials/salts), with consequent formation of segregation (frost-like tracery) patterns. The cryofixation quality of high pressure-frozen animal tissue has previously been assessed indirectly by morphological inspection for the absence or presence of these manifestations. However, it is now possible to evaluate cryofixation quality directly by electron diffraction analysis of frozen hydrated sections (see Fig. 1a, b).

In the forerunner of the current study (Studer et al. 1995), we systematically analysed the cryopreservation quality of high pressure-frozen young adult bovine articular cartilage tissue by electron diffraction analysis of cryosections, and established under standard and reproducible conditions the depth to which true vitrification occurred within each zone of 150-μm tissue slices. Using these same conditions, we here undertook an ultrastructural analysis of young adult bovine articular cartilage extracellular matrix using unstained cryosections. In order to be able to compare the new results with those obtained more commonly using adopted approaches, a portion of the high pressure-frozen material was freeze-substituted in acetone and embedded for conventional (rather than cryo-) transmission electron microscopy, tissue sections being derived from those locations which were known (on the basis of electron diffraction analysis of crysections) to be truly vitrified.

Ultrastructural examination of vitrified extracellular matrix revealed a number of novel features. In both cryosections and freeze-substituted/embedded material, cross-sectioned collagen fibrils manifested a pale body and a darker peripheral ring; profiles were, in turn, surrounded by a pale perifibrillar halo. The former two phenomena reflect differences in density, which are enhanced by phase-contrast imaging under defocused conditions, in the case of cryosections, and are in some way linked to the nature of the substitution medium, in the case of freeze-substituted/embedded tissue. The perifibrillar halo may represent a water-rich microenvironment (Studer et al. 1996).

Within cryosections, longitudinally sectioned collagen fibrils manifested a regular cross-banded pattern in vitrified and very thin cryosections (estimated thickness 50–80 nm, defocus setting 3–5 μm). Cryosections of crystalline tissue usually expand in thickness (100–120 nm), rendering phase-contrast imaging difficult. The periodicity revealed in vitrified cryosections, as well as in freeze-substituted/embedded (and stained) material, was the usual one of approximately 67 nm (fainter intermediate subbands were also seen). The periodic banding pattern manifested depends upon the contrast formation mechanism; in unstained cryosections it is generated mainly by phase contrast, whereas in stained freeze-substituted/embedded collagen fibrils it reflects differences in molecular staining affinity.

With respect to the distribution of collagen fibrils, these structures were sometimes encountered within the pericellular matrix compartment, which has hitherto been believed to be free of such components. A network of fine filaments (10–15 nm in diameter) was also observed, both here and throughout the entire extracellular substance (albeit at differing densities) of freeze-substituted/embedded tissue. The material composition of these elements remains to be elucidated, but the observation that many of the filaments manifested a periodicity similar to that of collagen fibrils is strongly suggestive of their being collagenous in nature; the possibility of their being beaded (hyaluronic acid) filaments must also be considered.

In vitrified freeze-substituted/embedded tissue, the proteoglycan-rich interfibrillar spaces did not exhibit a structurally identifiable correlate for the fine reticular network previously described (Arsenault and Kohler 1994; Engfeldt et al. 1994; Hunziker 1993; Hunziker and Schenk 1984); in the light of the present freezing quality data, this manifestation must now be regarded as a segregation artefact induced by ice crystal formation and growth, as suggested by Keene and McDonald (1993). If proteoglycans do form a filamentous network, as would be suspected on the grounds of their model molecular structure, then its dimensions lie below the resolution limits currently attainable by transmission electron microscopy of ultrathin sections. This may be partially attributable to the non-availability of molecule-specific staining compounds and also to the loss of lateral resolution caused by the high staining affinity of the protein-rich environment; there is no reason to suppose that these macromolecules lose their native structural organisation in vitrified tissue (Mayer and Astl 1992).

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