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Light microscopy evaluation of polyurethane vascular grafts porosity by Sudan Black B staining

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Summary

In small-diameter vascular grafts, the porosity of the internal surface plays an important role because it affects initial thrombus deposition and therefore the graft's patency. As well as many other studies reported in the literature we have carried out a study of the relationship between porosity and the manufacturing parameters of polyurethane (PU) grafts by standard scanning electron microscopy (SEM) analysis. However, SEM was not completely satisfactory for evaluating the 'sponge-like' uptake of water by the graft due to the unavoidable water loss and metal coating during preparation. In fact this preparation produces artefacts of the three-dimensional porous structure. To avoid this problem we investigated the possibility of observing the graft's internal surface through a stereomicroscope after it had absorbed water. We looked into a simple staining procedure which preferentially colours the PU graft fibres with respect to the void areas. After testing different kinds of stains, we eventually found that Sudan Black B, which usually stains for all kinds of lipid, turned out to be an excellent stain for the water-loaded PU grafts when diluted with ethanol. This staining procedure, coupled with a computerized image analysis system, allowed us to evaluate the degree of void and average void size of the graft internal surface and to correlate these data with graft density and manufacturing parameters.

Introduction

A major problem in vascular reconstruction procedures is the development of thrombi when blood contacts artificial surfaces, especially in small-diameter vascular grafts (SDVGs) (Bos *et al.*, 1998). Research work has demonstrated that the permeability and long-term wound healing characteristics of an SDVG are primarily affected by the porosity of its structure and by the chemical nature of the material used for its fabrication (Okoshi *et al.*, 1991, 1993, 1996). Porosity of the prosthesis wall in SDVGs plays an important role because it allows an initial deposition of a thin layer of fibrin along the luminal surface. This is later stabilized by pannus ingrowth of fibrous tissue and endothelium from the host vessel and by fibrous tissue and capillary ingrowth from periadventitial tissue through the microporous wall (Wesolwsky *et al.*, 1968). It has been reported that, for vascular grafts, pores should be small enough to limit transmural haemorrhage and to prevent periprosthetic haematoma, but they must be large enough to allow tissue ingrowth (Salvatore *et al.*, 1961). For soft tissue ingrowth, a pore size in the range 100–200 μm seems to be suitable (Taylor & Smith, 1972). Our research work focused on the study of optimal porosity characteristics of SDVGs manufactured by a 'spray, phase-inversion' (SPI) process, which is able to create different porous structures along the thickness of the graft wall (Soldani *et al.*, 1992). As working materials we used polyurethanes (PUs) that, because of their intrinsic good mechanical and blood-compatibility properties, have already been used for manufacturing porous or filamentous compliance conduits suitable as artificial vessels (White *et al.*, 1972; Annis *et al.*, 1978; Lyman *et al.*, 1978; Gogolewski & Pennings, 1982; Hess *et al.*, 1983; Leidner *et al.*, 1983; Wilson *et al.*, 1983). Grafts fabricated by the SPI process display a three-dimensional, sponge-like water-loaded, porous structure which needs special care for microscopical observations. To provide a detailed view of this fine porous structure we investigated the possibility of observing the graft with simple light microscopy while it is loaded with water and its spongy structure is completely expanded as if it were in physiological conditions. We found that Sudan Black B, which is known as a histological stain for all kinds of lipids (Pfüller *et al.*, 1977), shows strong affinity for the PU fibres of the graft and provides good contrast for microscopical observations. This paper reports the staining procedure and the results of the evaluation of the degree of void and average void size of the internal surface of Sudan Black B-stained grafts.

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Materials and Methods

Material preparation

An original 13% w/v PU solution in 2 : 1 THF/Dioxane (Cardiothane 51®, Arrow International, Everett, MA, U.S.A.) was diluted with the same solvent to obtain PU solutions of 0.2, 0.7, 1.2 and 1.7% w/v concentration. PU solutions to be used in the SPI process were made thermodynamically unstable and therefore ready to precipitate by a controlled addition of distilled water (used as a non-solvent) to the PU solution.

Grafts sample preparation

Porous grafts were made using the SPI technique (Soldani *et al.*, 1992). Briefly, the thermodynamically unstable PU solution and water were simultaneously, but separately, sprayed by two spray-guns onto a Teflon® rotating mandrel. Depending on the PU solution concentration and percentage of added non-solvent, different porous structures were obtained. Because this study relates to the evaluation of the average void size and degree of void of the graft internal surface, samples were prepared with a wall thickness of about 100 μm instead of the normal full graft preparation procedure, which involves the manufacturing of grafts with a wall thickness of about 400 μm. Making thinner samples reduced the fabrication time, the amount of material employed and facilitated microscopical observations under dark-field illumination.

Scanning electron microscopy

Graft samples were dehydrated in a graded ethanol series (20, 40, 60, 80 and 100%), then samples were critical point dried, from 100% ethanol (Balzers Critical Point Dryer CPD 030, Balzers, Milan, Italy), with carbon dioxide. Dried samples were mounted on scanning electron microscope specimen stubs and coated with gold in a sputter apparatus (Edwards Sputter Coater S150B, Edwards, Irvine, CA, U.S.A.). Samples were then examined and photographed using a Jeol 5600 scanning electron microscope.

Staining solution preparation

Sudan Black B (Carlo Erba, Milan, Italy) was dissolved in absolute ethanol to obtain a 0.3% (w/v) solution. The solution was stirred for 2 h at room temperature and then poured into well-stoppered dark bottles. In these conditions the solution remained stable for 1 month. We recommend filtering the solution before use.

Staining procedure

Grafts were cut into 6 mm length pieces without being removed from the Teflon mandrel (3.5 mm OD). The thin layer of material was easily cut lengthways with a lancet, glued with adhesive tape over a histology glass slide, dried with blotting paper, stained with 20 μL of Sudan Black B at room temperature, rinsed with distilled water, and then covered with a cover glass. Because of the insolubility of the Sudan Black B in water, grafts, once stained, can be stored in distilled water for later use.

Light microscopy and image analysis

Stained samples were observed using a stereomicroscope (SZH10, Olympus, Milan, Italy) at 70× magnification under dark-field illumination. Images were acquired by a video camera (KY-F32, JVC, Milan, Italy) and analysed by a computerized image analysis system (KS 300–3.0, Zeiss, Jena, Germany). Images were further processed by both an automatic and manual procedure. In the first case, after black and white conversion, images were 'binarised' by electronic elaboration, then the software calculated the percentage of white (void) and black (full) areas with respect to the total area. In the second case, circles were manually drawn following the perimeter of visible void areas, then the software calculated the average void diameter, perimeter and area.

Density measurement

Graft density was determined at room temperature using a kit for density determination of liquids and solids (Sartorious YDK01, Göttingen, Germany) for use with a precision balance (Sartorious BP221S). The kit is based on the Archimedes' principle. Analysed graft samples had an internal diameter of 3.5 mm, a wall thickness of 100 μm, and were 10 mm long. The following formula has been used for density calculation:

$$\rho = (W_a \times \rho_{fl})\, W_a - W_{fl}$$

where W_a = weight of solid in air, measured with the precision balance, W_{fl} = weight of solid in immersion, measured by the kit, ρ_{fl} = density of fluid in H_2O (density = 1).

Results

Figure 1(a) shows a scanning electron photomicrograph of the internal surface of a highly porous graft made with a 0.2% PU solution to which 17% of distilled water was added. The severe shrinkage and collapse of the graft, which made analysis of the porous structure impossible, is clearly visible. On the other hand, light microscopic observation of the same unstained graft samples loaded with water, both in bright and in dark field, did not provide enough contrast to give a clearly defined image and the porous structure of the graft internal surface was weakly outlined (Fig. 1b). In contrast, Sudan Black B-stained samples obtained from different concentrations of PU solutions showed clearly defined intact internal surfaces of various porosity, allowing an easy identification of void with respect to total area in the

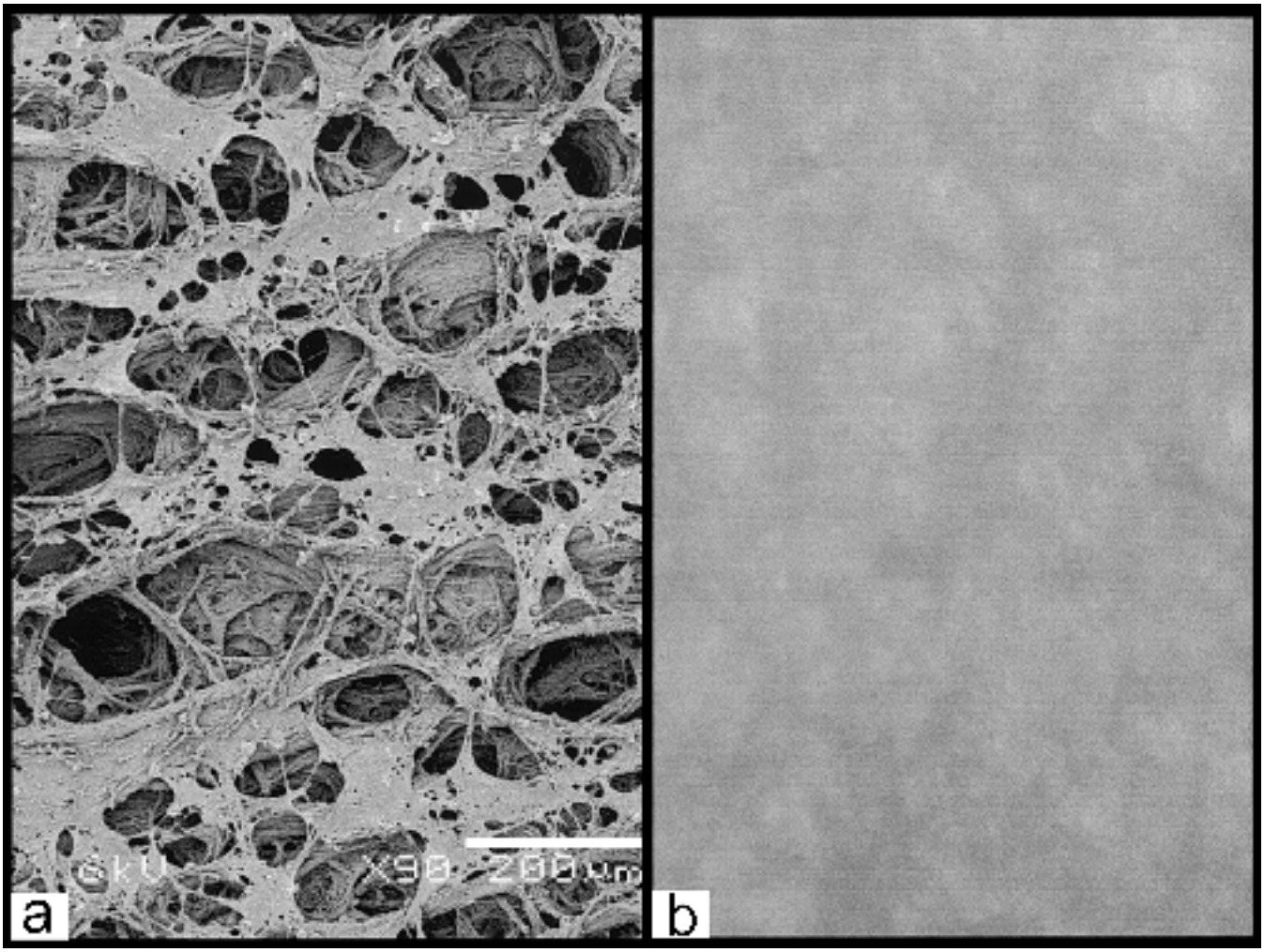

Fig. 1. (a) Scanning electron microscopy image of the internal surface of a highly porous graft sample made by a 0.2% w/v PU solution +17% v/v of H_2O. The graft porous structure is not well outlined and diffuse artefacts due to shrinkage and collapse are visible. Original magnification 90×, marker = 200 μm; (b) light stereomicroscopy image of the internal surface of an unstained highly porous, water-loaded, graft sample made by a 0.2% w/v PU solution +17% v/v of H_2O. The polygonal graft porous structure is weakly outlined and porosity measurements are not possible. Original magnification 70×, dark field.

stereomicroscope (Figs 2(a)–(d)). Automatic image processing and analysis of Sudan Black B-stained samples allowed the degree of void of the graft internal surface to be evaluated (Fig. 3). In Fig. 4 the relationship between percentage of degree of void and polymer concentration is reported. The diagram shows that a degree of void of about 60% can be obtained with a polymer solution of relatively low concentration (0.2% w/v), whereas a degree of void of about 14% can be obtained with a polymer solution of relatively high concentration (1.7% w/v). Data interpolation shows a linear trend between variables with a correlation coefficient of 0.98, which indicates that intermediate data can be extrapolated with good accuracy. Figure 5 illustrates the relationship between measured graft density and percentage of degree of void. The diagram shows that the density of a graft with a degree of void of about 60% approaches the density of water (1 g mL^{-1}), suggesting that the void volume of the graft is completely filled with water. By contrast, a graft featuring a degree of void of about 14% shows a density value 26.5% higher than that of water, implying that much less water can enter a closer graft structure. Also in this case the data interpolation shows a linear trend between variables with a correlation coefficient of 0.93, which indicates that intermediate values can be extrapolated to a good approximation. Manual image processing and analysis of Sudan Black B-stained samples permitted evaluation of the average void size of the graft internal surface (Fig. 6). The relationship between the percentage of water added to the polymer solution and the average void size is shown in Fig. 7. The diagram shows a non-linear trend between variables, which can be attributed to intermolecular forces and critical phase-separation phenomena, responsible for the ultimate graft porosity occurring in the polymer solution as a result of the gradual non-solvent addition (Kestin, 1985). It can be observed that, up to a

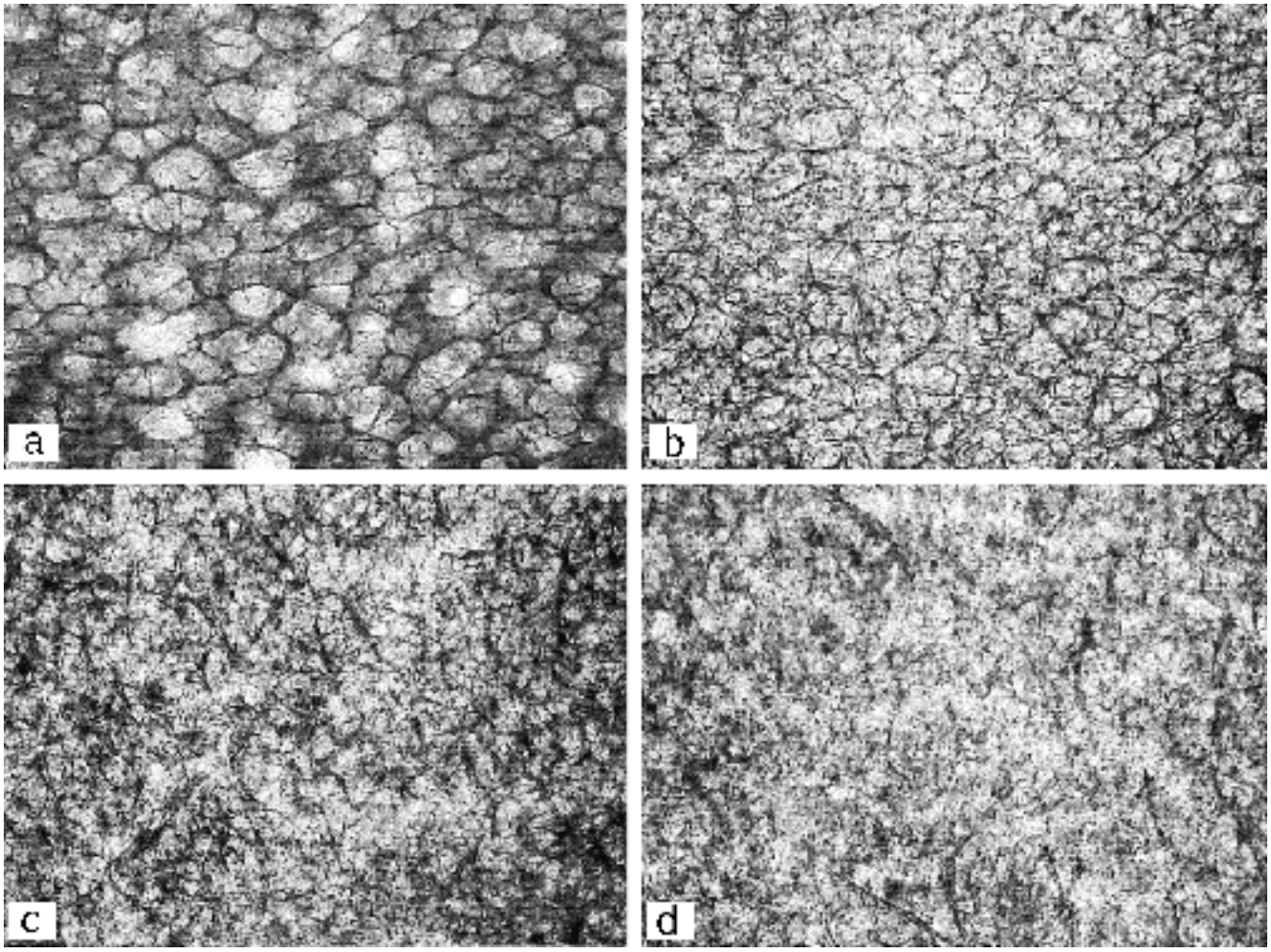

Fig. 2. Light stereomicroscopy images of the internal surface of Sudan Black B-stained, water-loaded, graft samples made by the following solution compositions: (a) 0.2% w/v PU solution +17% v/v of H_2O; (b) 0.7% w/v PU solution +17% v/v of H_2O; (c) 1.2% w/v PU solution +17% v/v of H_2O; and (d) 1.7% w/v PU solution +17% v/v of H_2O. It can be seen that grafts made with a relatively low PU solution concentration (0.2%) show a typical highly porous polygonal structure with large voids (a). Grafts made with an intermediate concentration (0.7%) show the same polygonal structure but with smaller voids (b), whereas grafts made with relatively higher concentrations (1.2% and 1.7%) show much closer structures ((c) and (d)), 1.7% being the closest structure of the series (d). Original magnification 70×, dark field.

relatively low water addition (10%), the average void size slowly varies from about 60 ± 17 μm to 90 ± 8 μm. On adding more water, a rapid increase of the average void size up to a maximum of 173 ± 21 μm (corresponding to a water addition of 17%) can be observed. We found that 17% of water added to a 0.2% w/v PU solution resulted in an optimal polymer/solvent/non-solvent ternary system able to provide the largest, well-outlined polygonal voids. Moreover, this value correlates with the highest percentage of degree of void shown by the internal surface of grafts made with this ternary system (see Fig. 4). A further addition of water up to 19% resulted in an abrupt decrease of the average void size, and microscopical observations showed that this correlated with disruption and collapse of the graft's three-dimensional structure.

Discussion

Prior to our discovery that Sudan Black B displays a strong affinity for the sponge-like structure of polyurethane grafts, we had used standard SEM in attempting to evaluate graft porosity (Okoshi *et al.*, 1991, 1993, 1996). However, a major drawback of SEM was that the sample preparation procedure, involving water removal and metal coating, causes artefacts of the graft and thus prevents effective detailed observations and measurements of the porous structure. To avoid this problem, we directed our research towards developing a simple light microscopy method of evaluating the internal surface microstructure of the graft in its native state. At first we tried two common light microscopy stains – methylene blue and crystal violet – but both

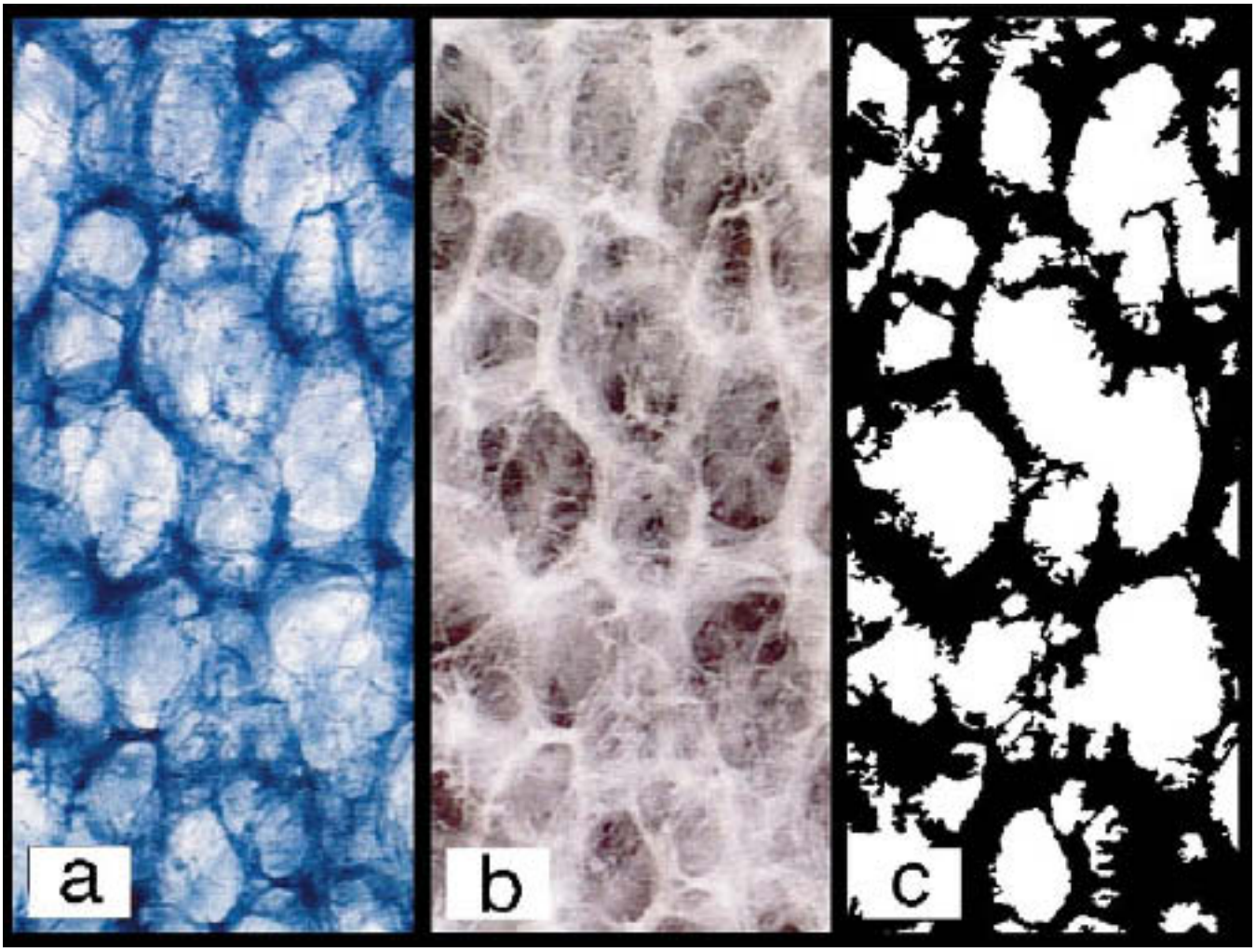

Fig. 3. Representative automatic image analysis elaboration of a Sudan Black B-stained graft sample made with a 0.2% w/v PU solution +17% v/v of H_2O: (a) image acquired by the video camera through the stereomicroscope; (b) image after colour inversion; (c) image 'binarised' by electronic elaboration. At the end of the procedure the degree of void of the graft internal surface [percentage of white (void) and black (full) areas respect to the total area] was automatically calculated by the computerized image analysis system.

of them stained the whole surface lightly with no contrast and, furthermore, they were dissolved by water during the preservation. Commercial black inks, such as Indian ink, acrylic and oily inks (pure or diluted in various solvents, heated and/or agitated) were tested, but the results and the reproducibility were not satisfactory because the staining produced dispersed spots on the whole surface. After these tests we investigated stains able to give more selective contrast (black or blue) to fibres with respect to void areas and we found that Sudan Black B yielded optimal results for this purpose. Stained grafts showed a specific stain intensity in the blue–green range, giving high contrast between fibres and voids without noticeable artefacts. We hypothesize that the graft staining mechanism might be related, first, to a mild PU material swelling due to the ethanol solvent of the stain and, second, to a dissolution of the stain into the PU matrix with consequent establishment of ionic interactions with atoms of the polymer backbone. To demonstrate these interactions, a specific investigation of the interactions between the PU and the Sudan Black B stain is in progress with the aid of differential scanning calorimetry (DSC) and infrared attenuated total reflectance (IR-ATR) analyses.

The Sudan Black B staining technique, together with computerized image analysis, allowed us to study the effect of two important manufacturing parameters involved in the SPI material processing: (1) polymer solution concentration and (2) volume of the non-solvent added to the polymer solution. These parameters were found to determine the final graft porous structure and to correlate with graft density measurements.

Other authors report that Sudan Black B stain has been used as a tool for histological evaluation of implanted polymeric biomaterials embedded in glycol methacrylate. These authors report that polymeric implants stained very specifically with Sudan

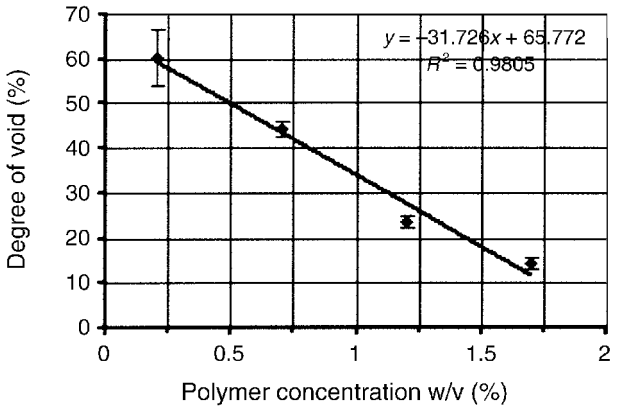

Fig. 4. Relationship between percentage of degree of void and polymer concentration. Data were obtained by automatic image analysis elaboration of the Sudan Black B-stained grafts shown in Fig. 3. Each data point is the mean of four samples and vertical error bars are standard deviations. The degree of void shows a linear regression respect to polymer concentration with $R^2 = 0.98$.

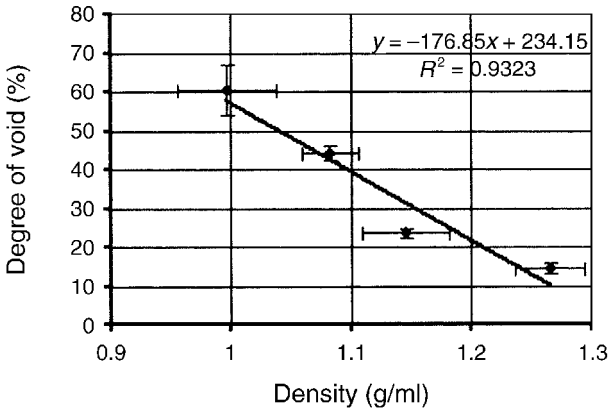

Fig. 5. Relationship between measured graft density and percentage of degree of void. Data about the degree of void are those reported in Fig. 4, whereas density data were obtained by three weight determinations and horizontal bars are standard deviations. The degree of void shows a linear regression respect to density with $R^2 = 0.93$.

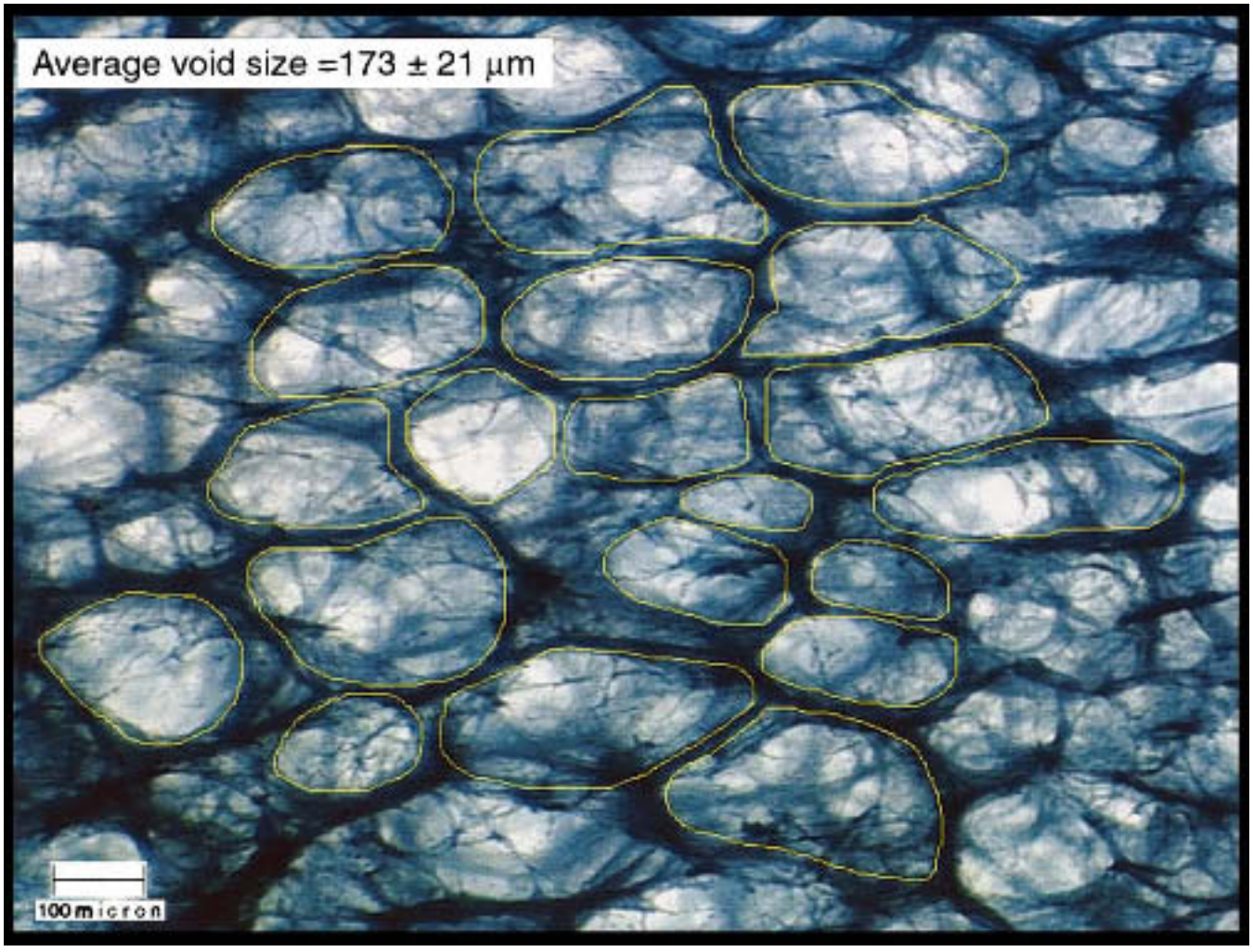

Fig. 6. Representative manual image analysis elaboration of a Sudan Black B stained graft sample made with a 0.2% w/v PU solution +17% v/v of H_2O. Images were acquired by the video camera through the stereomicroscope and circles were drawn manually following the perimeter of visible void areas. Average void size was calculated by image analysis software and was 173 ± 21 µm. Original magnification 70×, dark field, marker = 100 µm.

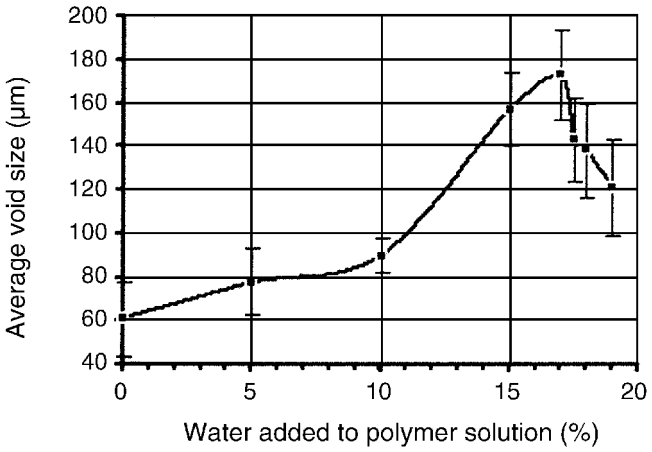

Fig. 7. Relationship between the percentage of water added to a 0.2% polymer solution and the average void size. A non-linear trend between variables is shown. Each data point is the mean of 20 random measurements and error bars are standard deviations. Large, well-outlined polygonal voids were obtained with a water addition of 17%.

Black B alone, and that this stain makes it possible to quantify the biodegradation process by image analysis (Hoeksma *et al.*, 1988). Moreover, it is suggested that the histological disappearance of the polymeric biomaterials could be used as a parameter for biodegradation (Hoeksma *et al.*, 1988). Based on these findings we can extrapolate that the Sudan Black B staining technique could also be used, following graft implantation experiments, to monitor the biodegradation, if any, of PU porous graft structure. In conclusion, the use of Sudan Black B staining for the evaluation of the porous structure of grafts by light microscopy, before and after implantation, appears to be a valuable aid not only for research on vascular grafts but on biomaterials in general and it may represent a valid alternative to the use of standard SEM.

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