A CHEMICAL, MORPHOLOGICAL AND BIOCHEMICAL COMPARATIVE STUDY OF COLLAGEN WOUND DRESSINGS

Taddei, A.R.[1], Picchietti, S.[2], Gambellini, G.[1], Fausto, A.M.2 and Giorgi, F.[3]

([1]) Interdepartmental Centre For Electron Microscopy (CIME), ([2]) Tuscia University, Viterbo, ([3]) Department of Neuroscience, University of Pisa

BIOPAD EQUINE COLLAGEN

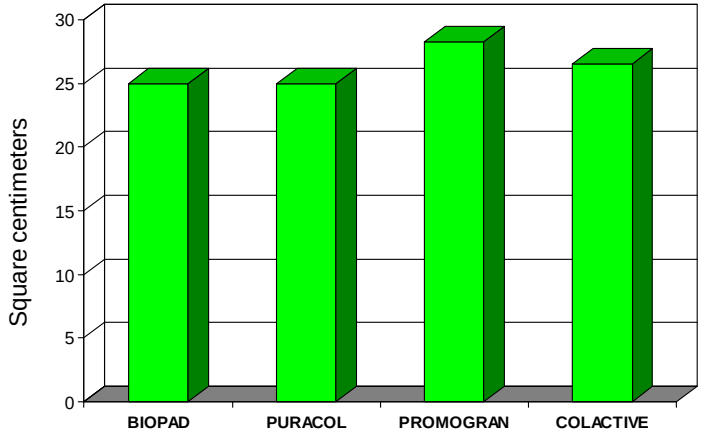

Table 1

The dimensional characteristics of the collagen samples examined in this study are reported.

Three of these, BIOPAD®, PURACOL® and COLACTIVE®, are squared in shape, while the fourth, PROMOGRAN®, is hexagonal in shape. BIOPAD® is very thick, while COLACTIVE® and PURACOL® are much thinner.

The overall surface is very similar for all collagen samples.

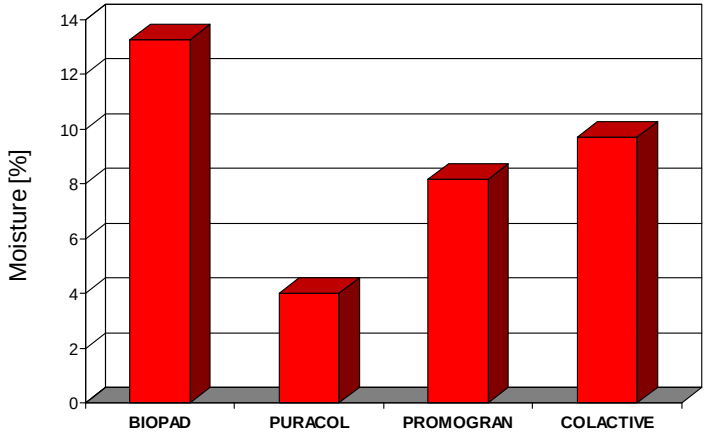

Table 2

The extent of weight losses obtained upon drying the collagen samples are reported: higher values are obtained for BIOPAD®, COLACTIVE® and PROMOGRAN®, while PURACOL® has an anomalous low water content, less than 10% which corresponds to a threshold value for a collagen standard of type I.

The extent of hydration is important for evaluating the nitrogen and hydroxyproline content of the dry substance, which is equal to the actual collagen content of the sample under analysis.

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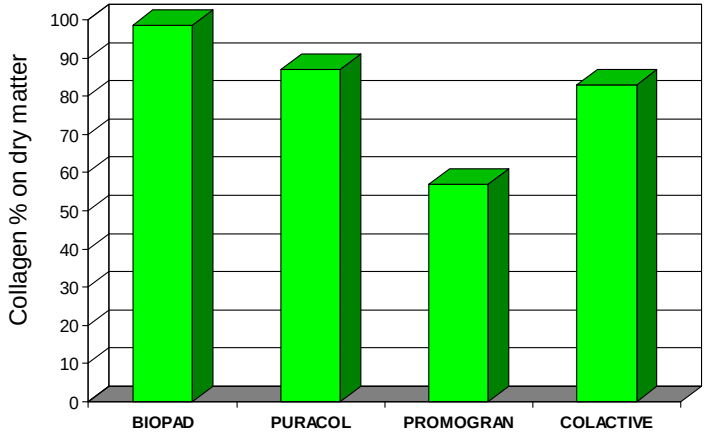

Table 3

The extent of nitrogen content for each collagen sample is reported. This value is correlated with that of the dry matter and hence with the value of the actual collagen content, through the use of the 5.50 conversion factor from nitrogen to the scleroprotein collagen.

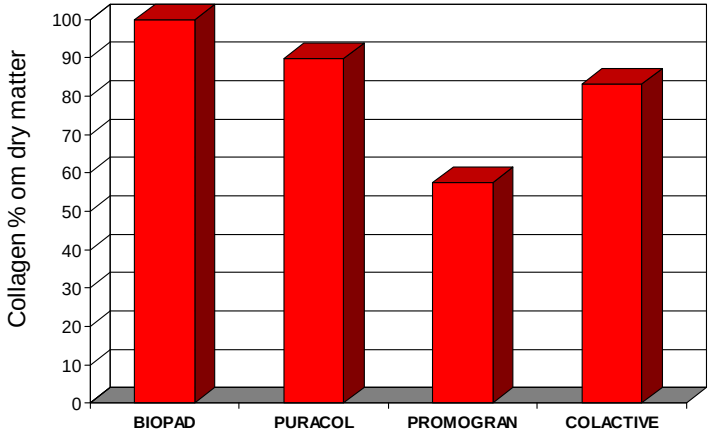

Table 4

The actual hydroxyproline content for each collagen sample is reported. Data are expressed as a percentage of the hydroxyproline content, with respect to its value on the dry matter and the value as collagen, obtained by using the 7.46 conversion factor from hydroxyproline to the scleroprotein collagen.

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BIOPAD EQUINE COLLAGEN

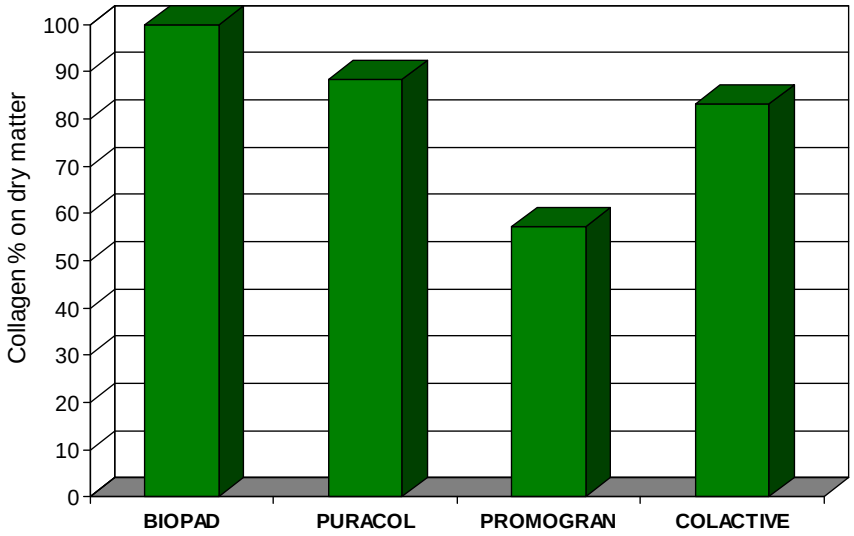

Table 5

Four collagen that are used as wound dressings have been tested in this study to evaluate their dimensional and chemical characteristics.

Three of these samples (BIOPAD®, PURACOL® and COLACTIVE®) exhibit such dimensional characteristics as surface and shape that are well intended to maximise their interaction with the wound, while the fourth sample (PROMOGRAN®) has an hexagonal shape and about 28 cm² of surface available for interacting with the wound.

In addition, BIOPAD®, COLACTIVE® and PROMOGRAN® have various degree of porosity (see later), while PURACOL® has a wafer thin consistency and a very dense mesh.

The knowledge of values related to the actual nitrogen and hydroxyproline contents for each collagen sample, together with their humidity content, allowed us to estimate the real content of dry collagen in each sample under test. This value expressed as a mean of two independent determinations, is reported in Table V and is about 100% for BIOPAD®, 88.4% for PURACOL®, 57.2% for PROMOGRAN® and finally 83% for COLACTIVE®.

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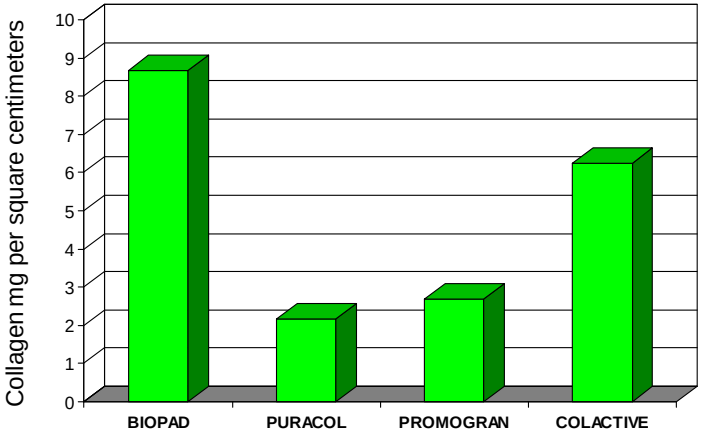

Table 6

To evaluate critically how much collagen is really in contact with the wound surface when the medical device is applied, the quantity of collagen per square centimeter has to be evaluated. This value may be calculated by considering the weight of the medical device less its water content and multiplied by the percentage of collagen present in the medical device in the form of dry matter. The resulting value is then divided by the pad surface.

As it can clearly be seen, the highest value for this parameter is obtained for BIOPAD® amounting to about 140% more than COLACTIVE®, about 320% more than PROMOGRAN® and finally about 400% more than PURACOL®.

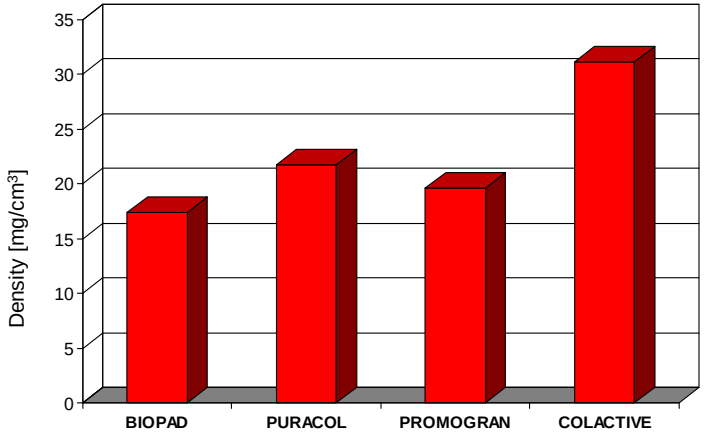

Table 7

There are however other criteria to be considered. A medical device applied on a wound has to absorb all haematic and lymphatic fluids present in the wound itself.

From this point of view the porosity of the collagen pad plays an important role and this may be estimated as the density of the dry collagen per unit of volume. This value may be calculated from the weight of the medical device less its water content. The value so obtained must then be multiplied by the percentage of collagen in the medical device, considered as a dry matter and finally the resulting value must be divided for the volume of the medical device.

The lowest density and then the highest porosity is to be associated with BIOPAD®, followed by PROMOGRAN®, PURACOL® and COLACTIVE®

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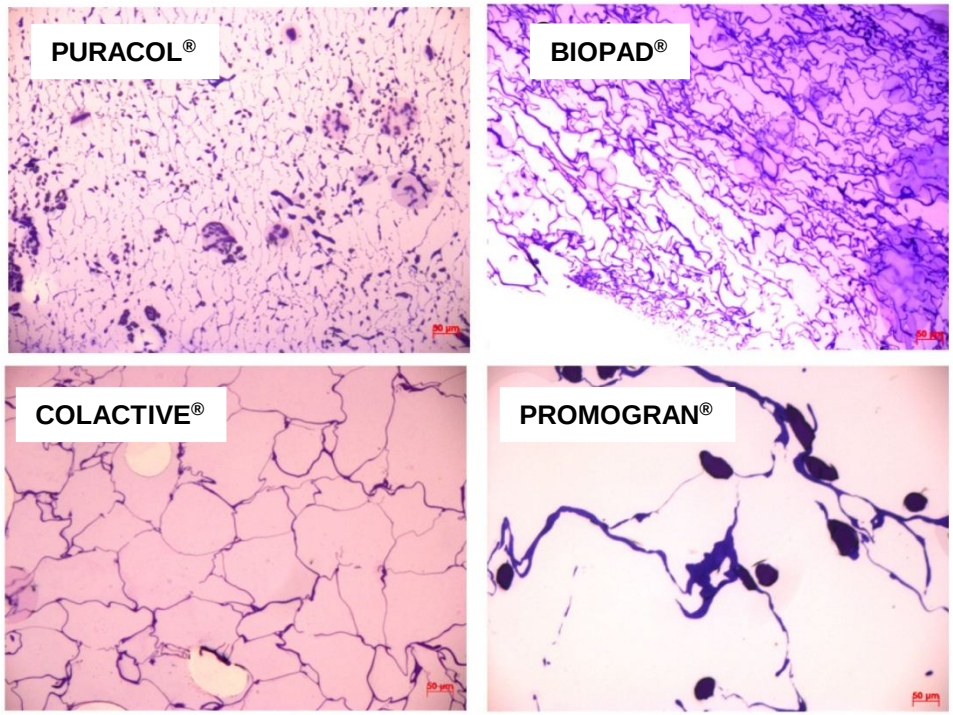
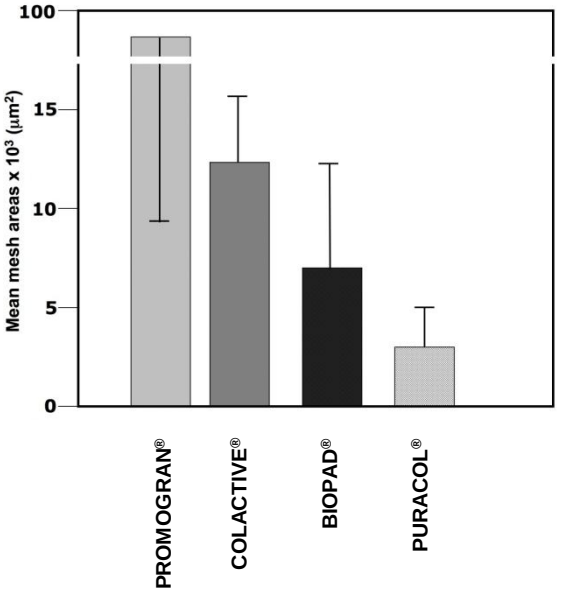


Fig.1 - Light microscope analysis

As it can be clearly seen, the four collagen samples exhibit different structural characteristics, as regards to the extent of interstitial areas and mesh properties. In particular, it is visually apparent that PROMOGRAN® and PURACOL® exhibit the highest and lowest strand extension, respectively, amongst all collagen samples examined in this study. COLACTIVE® and BIOPAD® have intermediate values amongst these extreme conditions, although the former has also thinner collagen strands as supports of its mesh structure. To provide a quantitative esteem of this visual impression, areas enclosed by the collagen strands were calculated for each sample through an image analyzer and the data obtained this way were reported on the histogram shown below (Fig. 2).

The statistical analysis reported in Fig.2 corroborates the idea that PROMOGRAN® has too high a value for the mesh area, indicating that in this sample there are too few collagen strands per unit of volume. The opposite condition is provided by PURACOL® which has the lowest mesh area due to thinner collagen strands, but more densely distributed within the same area, once again indicating a lower percentage of collagen dry matter per unit of volume. Finally, BIOPAD® and COLACTIVE® are both within more reasonable values for mesh areas, although only in the first sample this value is associated with thicker collagen strands.

Fig.2



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BIOPAD EQUINE COLLAGEN

Scanning electron microscope analysis

The analysis of a collagen sample by light microscopy suffers of a major drawback, that is to say, what in origin belongs to a volumetric extension is ultimately quantified on a bi-dimensional plane.

As a result, the structural characteristics that are envisioned under these conditions may not reliably reflect some of the real differences between the various samples being tested.

To overcome these difficulties the same collagen samples were analyzed by scanning electron microscopy that, as it is well known, provides a realistic view of the three-dimensional structure of the sample under examination.

For images obtained through these techniques to reflect a realistic comparison, collagen samples had to be fixed without any prior hydration – as otherwise the overall structural appearance of the collagen could be altered by the water intake – and only after that a critically point dried sample has been fractured – as otherwise the morphology of any newly exposed surfaces could be altered by pressure of the cutting edge of a running blade.

The figures that are shown below were taken in conformity with these criteria.

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Scanning electron microscope analysis - COLACTIVE®

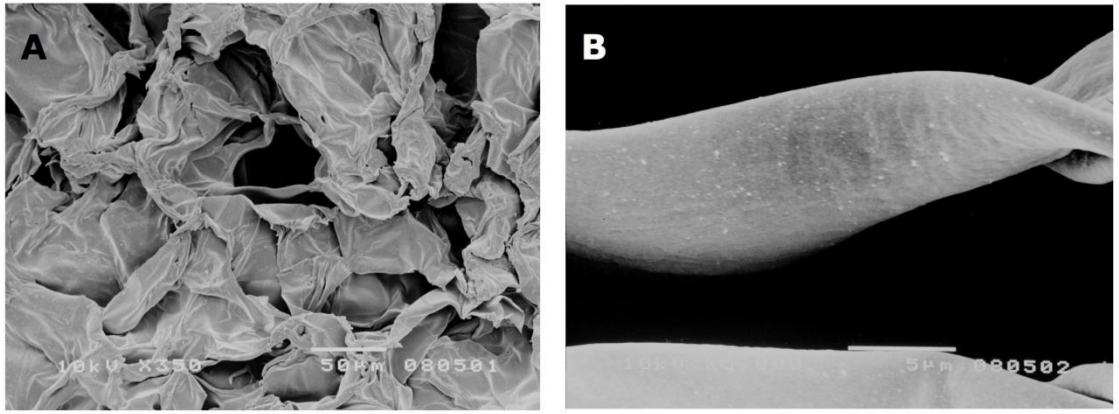

Fig 3. *Scanning electron micrographs of COLACTIVE® collagen as seen at low magnification (x350 in A) and at higher magnification (x15000 in B). Scale bars are indicated in the micrographs as 50 µm and 5 µm, respectively.*

Analysis of the COLACTIVE® sample by scanning electron microscopy shows that what appear as collagen strands by light microscopy in reality are very thin intermingled laminas (Fig. 3A). The extent by which interstices are meshed together is very high, but the collagen strands that delimit this interlaced structure are very thin amounting to something less than 2 µm in thickness (Fig. 3B).

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Scanning electron microscope analysis - BIOPAD®

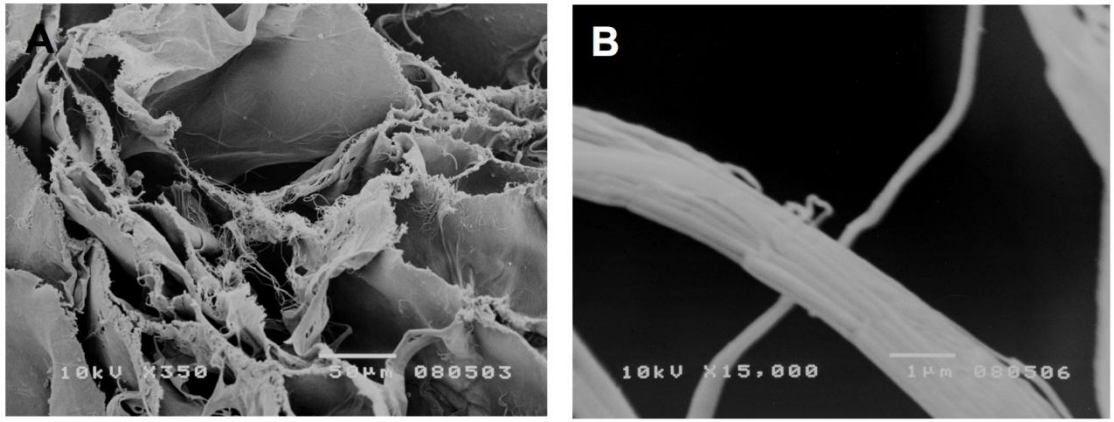

Fig 4. *Scanning electron micrographs of BIOPAD® collagen as seen at low magnification (x350 in A) and at higher magnification (x15000 in B). Scale bars are indicated in the micrographs as 50 µm and 5 µm, respectively. Note that laminas are highly complex in structure due to the fact that appear made of several densely packed collagen fibres.*

Fig. 4A is a low magnification picture of the BIOPAD® sample. The three-dimensional extensions of the collagen strands in this sample shows that laminas are more complex in structure, than envisioned at first sight by simple light microscope observations. This demonstrates that collagen laminas in BIOPAD® samples are formed by several collagen fibres tightly and densely packed together. Fig. 4B shows a detail at high magnification of one of these fibres as it leans out from an underneath lamina. At this enlargement it can be clearly seen that even this fibre has an underlining structure made up of numerous fibrils.

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Scanning electron microscope analysis - PROMOGRAN®

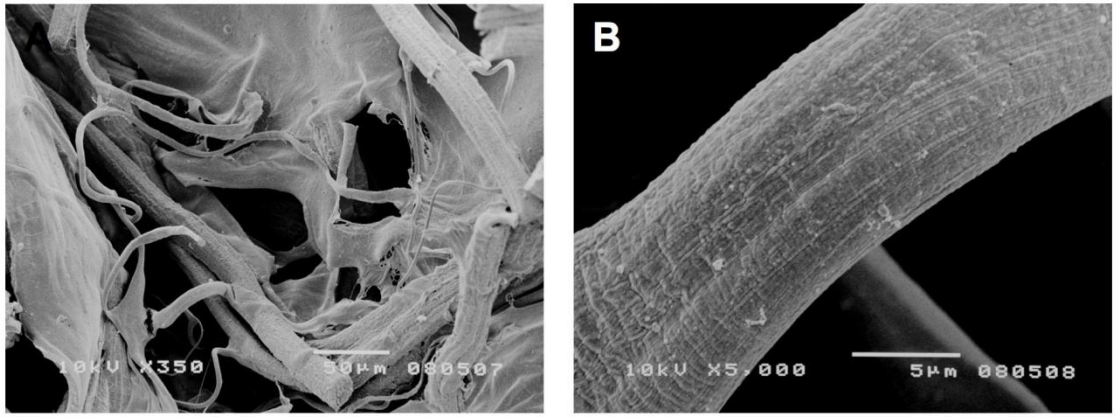

Fig 5. *Scanning electron micrographs of PROMOGRAN® collagen as seen at low magnification (x350 in A) and at higher magnification (x5000 in B). Scale bars are indicated in the micrographs as 50 µm and 5 µm, respectively. Note that a complex substructure can only be evidenced in the higher magnification picture of the collagen fibres.*

Fig. 5A is a low magnification micrograph of the PROMOGRAN® sample showing several collagen laminas and fibres dispersed within the mesh substructure. What this image shows is that very few laminas are fractured crosswise, indicating and thus confirming that there are too few collagen strands per unit of volume. When examined at higher magnification, the collagen fibres in this sample exhibit a molecularly complex substructure associated with the thinner fibrils (Fig. 5B)

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Scanning electron microscope analysis - FIBRACOL®

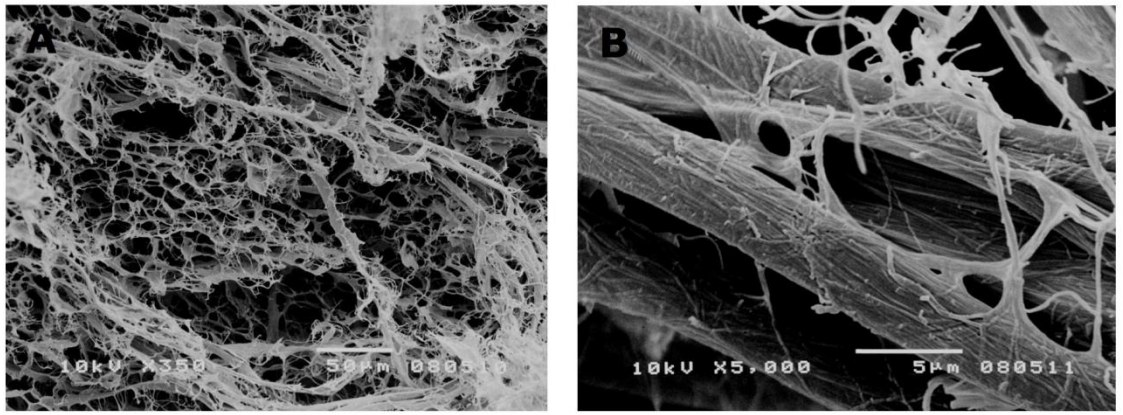

Fig 6. *Scanning electron micrographs of PURACOL® collagen as seen at low magnification (x350 in A) and at higher magnification (x5000 in B). Scale bars are indicated in the micrographs as 50 µm and 5 µm, respectively. This is the only collagen sample in which the mesh infrastructure is evidently thicker.*

Finally, when PURACOL® was examined at the scanning electron microscope under similar experimental conditions, the first structural characteristic that could be easily envisioned was due to a high mesh density (Fig. 6A). For instance, comparison of all A pictures of Figs. 3-6 shows clearly that only PURACOL® has an abnormally higher mesh density. However, since this structural condition is also associated with thinner collagen strands, a higher mesh density does not necessarily correspond to higher collagen contents. On the contrary, the opposite condition is more likely to occur in this sample. At higher magnification PURACOL® shows that collagen laminas are so fragile that they are reduced to thin collagen fibrils cross-linking wider collagen bundles of fibres (Fig. 6B).

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BIOPAD EQUINE COLLAGEN

Transmission electron microscope analysis

It is well known to morphologists that x15,000 constitutes almost the maximum magnification at which a sample can be resolved by scanning electron microscopy. As we have already seen, these conditions are not good enough to resolve the black and white staggered bands of the collagen fibres.

On the other hand, it has been said in the introduction that this is a fundamental criteria by which collagen can be unambiguously identified. Thus to provide a final morphological evidence that all samples we have been studying in this period are truly collagen products, all samples have been processed for transmission electron microscopy in an attempt to achieve higher resolutions.

Under these experimental conditions, it is thus possible to verify whether collagen fibrils are properly assembled and, above all, if they exhibit a clear staggering pattern of black and white bands.

A CHEMICAL, MORPHOLOGICAL AND BIOCHEMICAL COMPARATIVE STUDY OF COLLAGEN WOUND DRESSINGS

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Transmission electron microscope analysis - COLACTIVE®

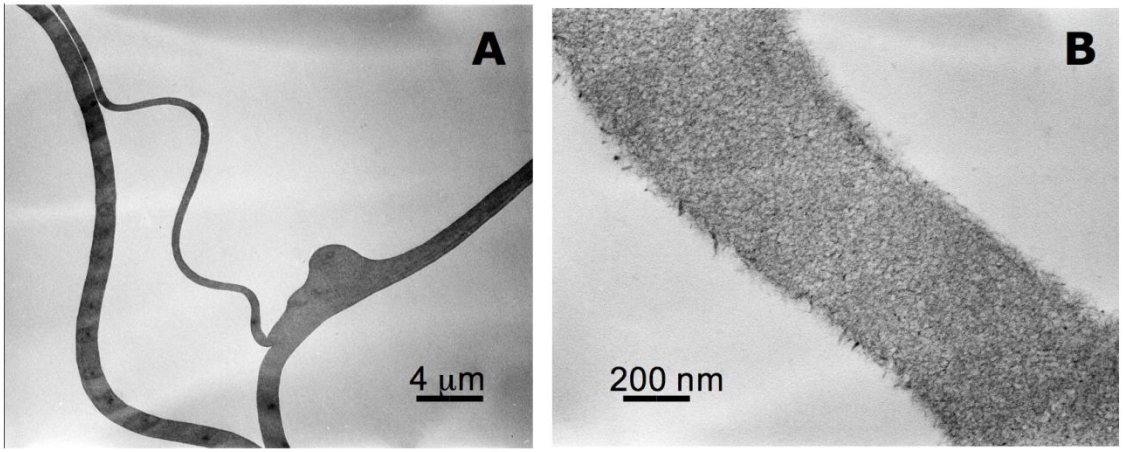

Fig 7. *Transmission electron microscope micrographs showing a low (A) and high (B) magnification sections of the COLACTIVE® sample. Scale bars of 4 µm and 200 nm are also indicated in the pictures.*

Fig. 7A is a low magnification micrograph of COLACTIVE® showing several collagen strands enclosing some mesh areas. From this picture one can obtain information concerning the actual thickness of the collagen strand. It is easy to observe that these strands thickness is less than 2 µm.
At higher magnification (Fig. 7B), the collagen strand does not reveal the presence of any banding pattern, thus indicating that either collagen fibrils are not present or, alternatively, that they have not been properly assembled during the extraction procedure and therefore it is not anymore native collagen.

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Transmission electron microscope analysis - BIOPAD®

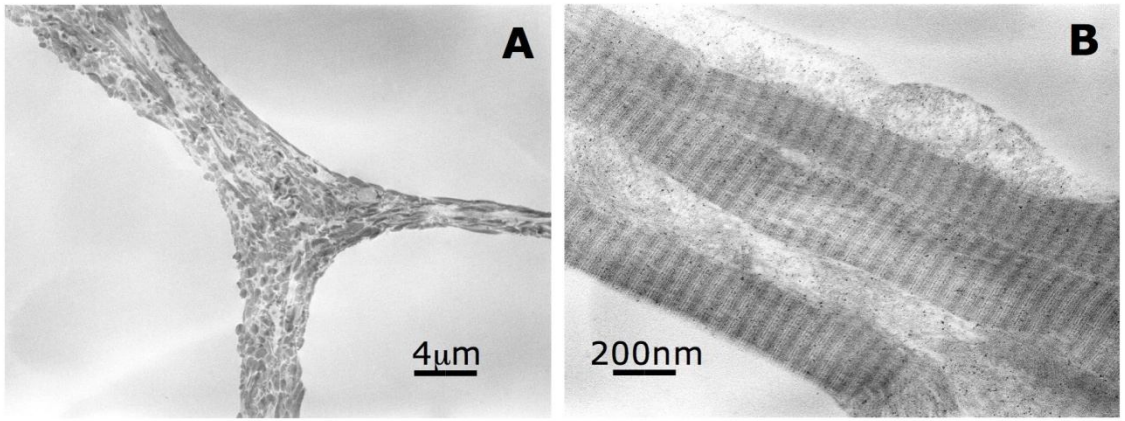

Fig 8. *Transmission electron microscope micrographs showing a low (A) and high (B) magnification sections of the BIOPAD® sample. Scale bars of 4 µm and 200 nm are also indicated in the pictures.*

On comparing Fig. 8A and 8A, one is struck by the finding that collagen strands in the BIOPAD® sample are much thicker – attaining up to 4 µm in thickness at certain joints – and above all, they exhibit a well defined substructure which is totally absent in the COLACTIVE® sample. At higher magnification the substructure that can be observed in the collagen strand is clearly due to the alternating pattern of black and white bands. Taken together, these observations suggest that collagen fibrils are not only present in due amounts to confer a proper thickness to the collagen strands, but they are also spatially arranged in such a way as to provide a densely packed mesh. These parameters may have a key role to play in relation to cell invasiveness of the collagen matrix and consequently in relation to the stability of the medical device on the wounded area.

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Transmission electron microscope analysis - PROMOGRAN®

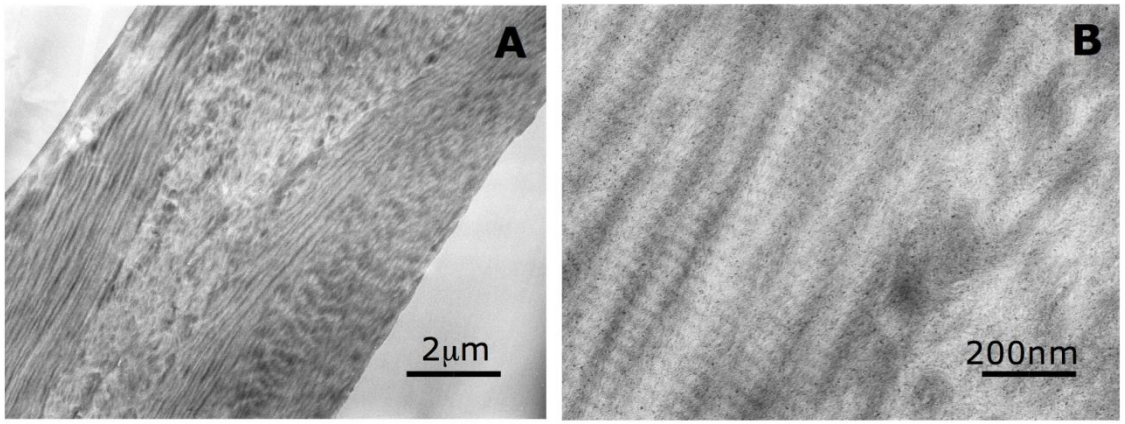

Fig 9. *Transmission electron microscope micrographs showing a low (A) and high (B) magnification sections of the PROMOGRAN® sample. Scale bars of 2 µm and 200 nm are also indicated in the pictures.*

When PROMOGRAN® samples were examined at the transmission electron microscope under similar conditions of hydration and sectioning, the first morphological characteristic that struck the observer's eye is the actual thickness of the collagen strands (Fig. 9A). Unlike those of COLACTIVE® and BIOPAD® that range to a maximum width of 2 and 4 µm, respectively, PROMOGRAN® was found to range in strand width from a minimum of 8 to a maximum of 12 µm. This is indeed a remarkable observation, especially if logically linked with the fact that PROMOGRAN® has also the largest mesh area of all collagen sample examined in this study.
These strands can be clearly shown to have typical collagen fibres with an alternate pattern of black and white bands (Fig. 9B). Taken all together these observations bring us to suggest that most of the collagen fibres in the PROMOGRAN® sample are densely packed in the collagen strands. However, just because of this dense packaging, the mesh area comprised between adjacent strands is bound to be too extended, and this may somehow affect the way this gel matrix interact with invading cells, which in turn may condition the actual gel persistence on the wounded area.

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Transmission electron microscope analysis - PURACOL®

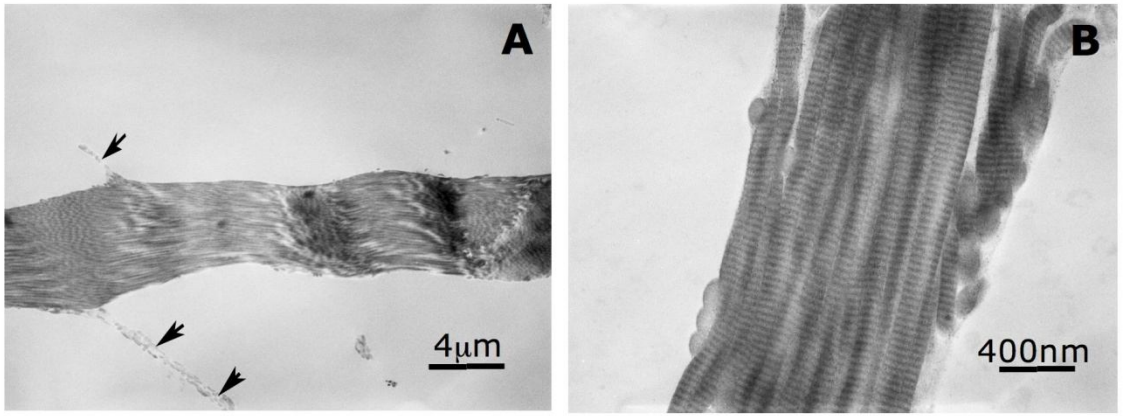

Fig 10. *Transmission electron microscope micrographs showing a low (A) and high (B) magnification sections of the PURACOL® sample. Scale bars of 4 µm and 400 nm are also indicated in the pictures.*

Finally when PURACOL® was examined by electron microscopy, both low and high magnification pictures revealed the presence of well staggered collagen fibres. It was apparent, however, that in low magnification pictures collagen was primarily associated with fibre bundles of about 4 or 5 µm in thickness. Other very thin fibrils could be seen branching out from these bundles and perhaps delimit the real mesh area of this collagen sample (Fig. 10A, see arrows). At higher magnification this collagen sample exhibits very nicely banded fibrils with a mean diameter of about 200 nm each. Altogether, these observations confirm our first impressions at the scanning electron microscope that PURACOL® has a highly dense mesh that is however bordered by thin collagen strands. The microscope analysis has in addition shown that, besides these thin collagen strands, there are also thick bundles of collagen fibres that are not involved in constructing the mesh bordering.

BIOPAD EQUINE COLLAGEN

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A CHEMICAL, MORPHOLOGICAL AND BIOCHEMICAL COMPARATIVE STUDY OF COLLAGEN WOUND DRESSINGS

Taddei, A.R.[1], Picchietti, S.[2], Gambellini, G.[1], Fausto, A.M.2 and Giorgi, F.[3]
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BIOPAD EQUINE COLLAGEN

Electrophoretic analysis

As stated in the introduction, the definition of such criteria as the presence of a banding pattern due to the staggered disposition of successive molecules should, in principle, be sufficient to identify collagen fibrils. However, it should also be observed that, while the presence of a staggered pattern is always a good indication for ascertaining the presence of collagen fibrils, the reverse condition may not always be true. It may in fact be possible that, under certain conditions, collagen molecules are prevented from assembling in a staggered manner. Thus, the final proof that all samples compared in this study do really contain collagen as a starting material, may only be provided by gel electrophoresis as the sole technique apt to resolve their polypeptide composition.

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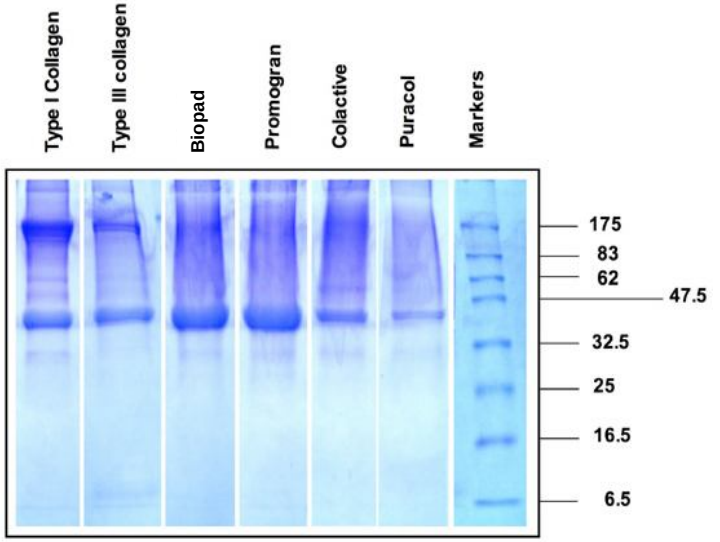
Several attempts were initially made to resolve electrophoretically all collagen samples following solubilization in Laemmli buffer. However, in spite of the numerous variations introduced into the extraction procedure, they always resolved into heavily stained smears and not in well defined fractions. In our views this may stem directly from the fact that all collagen samples are freeze-dried, a condition that may have caused extensive fibre cross-linking. These first attempts were thus followed by the adoption of an extraction procedure that made use of pepsin digestion as a preliminary step toward solubilization (see Material and Methods). Under these conditions, the electrophoretic analysis demonstrated that all samples were characterized by the presence of a major polypeptide fraction of about 40 kDa. Type I collagen treated according to the same protocol also proved to contain the same polypeptide. However, the COLACTIVE® sample had an additional polypeptide fraction of higher molecular weight (about 120-140 kDa) which is also shared by the collagen standard of type III.

These observations indicate that pepsin-treatment is likely to cause collagen solubilization by inducing partial cleavage of telopeptide regions in the assembled fibrils. By doing so, the enzyme may simply remove all pepsin-sensitive sites from the major α_1 and α_2 polypeptide chains and liberate only those fragments of the collagen sample that are resistant to the digestion. If this interpretation is correct, it is interesting to conclude that all samples behave in much the same way as type I collagen, indicating that they are likely to derive from tendon collagen. The sole exception to this conclusion is represented by the COLACTIVE® sample that shares a common electrophoretic pattern with type III collagen which is of dermal origin.



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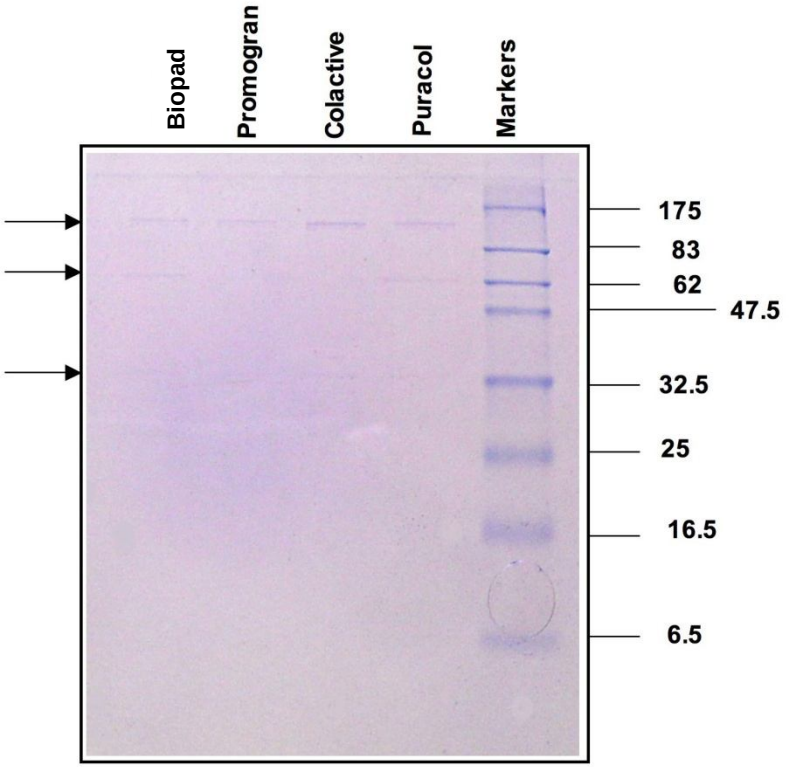
BIOPAD EQUINE COLLAGEN

To further test for the biodegradability of collagen samples, their susceptibility to collagenase digestion was critically evaluated. In the presence of collagenase, native helical collagens are cleaved simultaneously across all three chains or attacked as single strands (Seifter and Harper 1970). Under these conditions, all four collagen samples, i.e. BIOPAD®, PROMOGRAN®, COLACTIVE® and PURACOL® give rise to a major band of about 150 kDa plus other minor protein fractions of lower molecular weights. Due to the low concentration these proteins attain in our hands, it is difficult to ascertain any difference amongst collagen samples in their respective biodegradability to collagenase. The figure depicts the electro-phoretic resolution of collagen samples following collagenase digestion.



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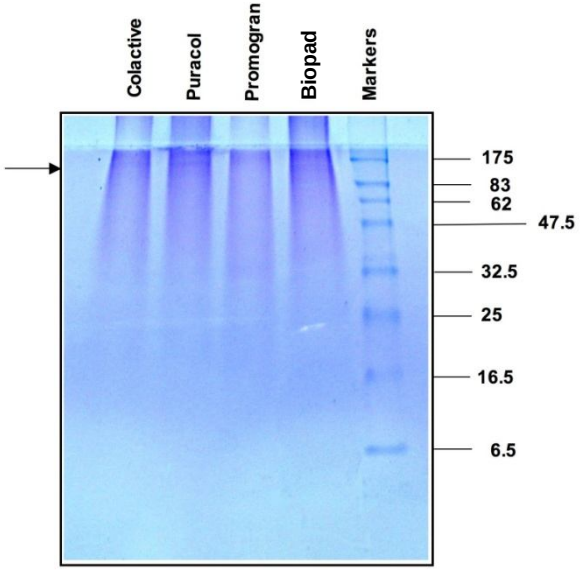
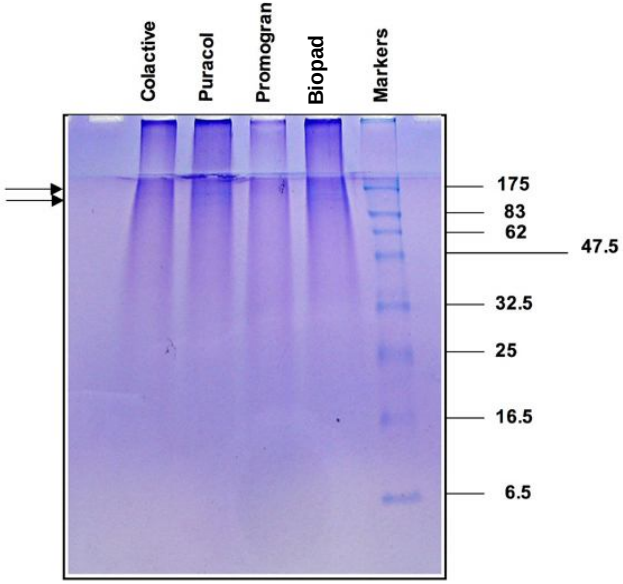
BIOPAD EQUINE COLLAGEN

To further address the question of biodegradability, collagen samples were exposed to metalloproteinase MMP-1 and MMP-13. Under these conditions, all four collagen samples appeared to yield a major 150 kDa protein fraction and an underneath diffuse smear, suggesting the possibility that the single strand polypeptide has been degraded into a series of smaller fragments. Once again comparison amongst these collagen samples does not allow us to draw any major conclusion concerning their biodegradability or their differential susceptibility towards MMP-1 (upper figure) and MMP-13 (lower figure) . One of the major difficulty in interpreting these results may be due to the fact that these samples are not water-soluble, most likely because the procedure employed for freeze drying makes them highly cross-linked. The correctness of this interpretation is testified by a number of reports showing that collagen scaffolds become dehydrothermally cross linked whenever exposed to high temperatures under vacuum. Under these conditions, the collagen network is not denatured into gelatine, but, on the contrary, it becomes stiff due to the formation of covalent cross links between the collagen chains (Yannas, 1972; Brien et al., 2004). If this interpretation is correct, then a major criterium one could use to ascertain the stability and/or biodegradability of the collagen scaffold could primarily be based on a morphological analysis, following exposure to various enzyme activities and/or cell types capable of providing these activities.





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BIOPAD EQUINE COLLAGEN

The Fig.11 demonstrate how the interlaced matrix of the collagen samples is modified following exposure to collagenase for 5 hours at 37 °C. As compared to the control pictures shown previously (see Fig. 1), exposure to collagenase causes both PURACOL® and PROMOGRAN® to become apparently deprived of the thinner threads present in the interlaced collagen matrix, thus retaining only some structurally denser spots. On the contrary, BIOPAD® retains the same overall structural characteristics, even though collagen threads are clearly frayed. Finally the COLACTIVE® samples become apparently totally collapsed into compact clumps.

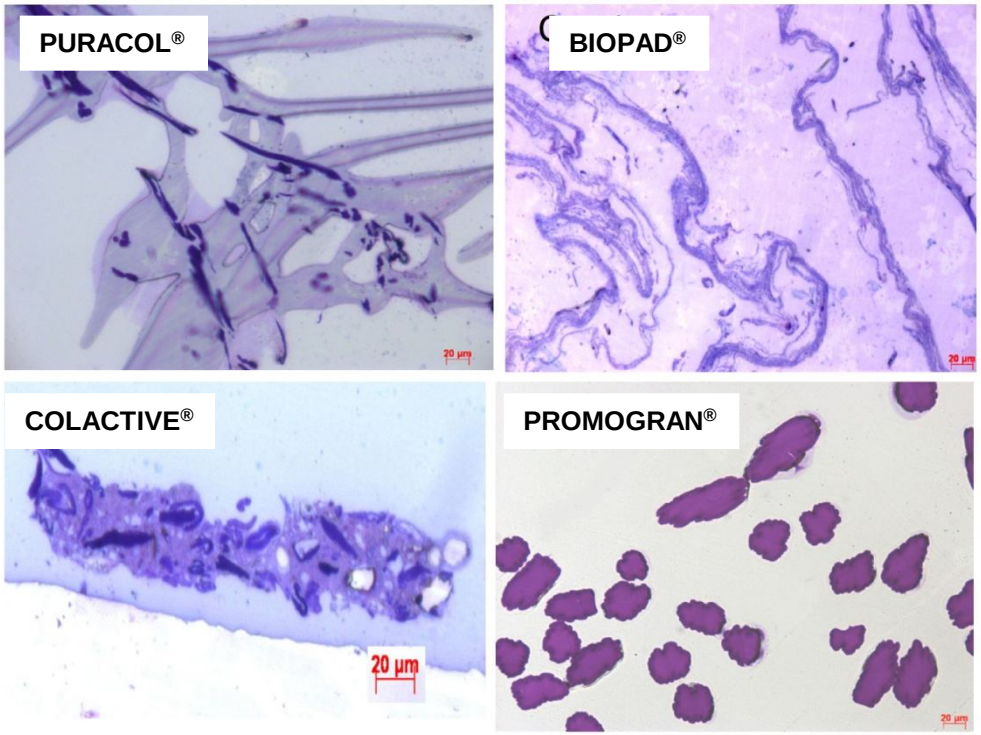

Fig. 11 – Light microscope views of thick sections from collagen samples embedded in Epoxy resins

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BIOPAD EQUINE COLLAGEN

To find out more about the structural modifications collagen samples undergo following exposure to collagenase, all collagen samples subject to collagenase exposure were also examined by electron microscopy. The Fig 12 shows several high resolution images of the PURACOL® collagen sample. These images refer to those high density threads that are still visible following removal of the thinner ones. As it can be clearly seen, they remain almost unaltered even after 5 h exposure to collagenase, suggesting that fibre-packaging may be so tight as to render it almost inaccessible to the enzyme.

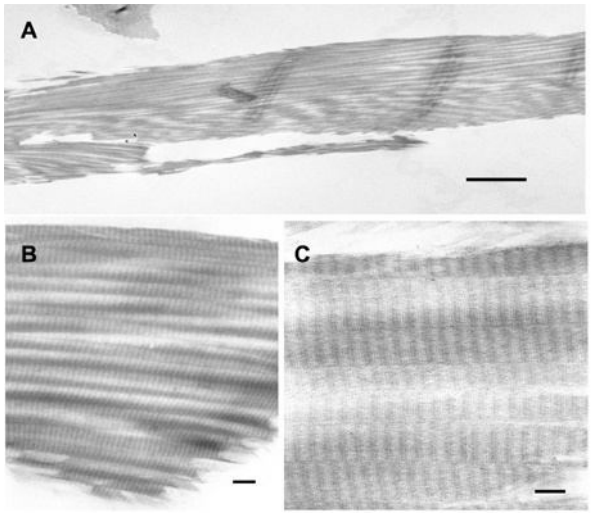

Fig 12 - High resolution images of PURACOL samples treated for 5 hours at 37 °C with collagenase. Scale bars are 2 µm (A), 200 nm (B) and 100 nm (C).

Unlike the PURACOL® samples, BIOPAD® was found to retain its overall structural characteristics as they are visible at low magnification. However, when frayed collagen threads were examined at higher resolution, they appeared fully disaggregated and much more dispersed than in control samples (Fig.13A). Interestingly, single collagen fibres are still very well resolved, suggesting that the primary effect of collagenase may be envisioned in the process of collagen disaggregation, rather than in fibril disassembly. Figs.13B and C show that isolated collagen fibrils from the BIOPAD® sample can still exhibit their characteristic periodicity in spite of their disassembled condition.

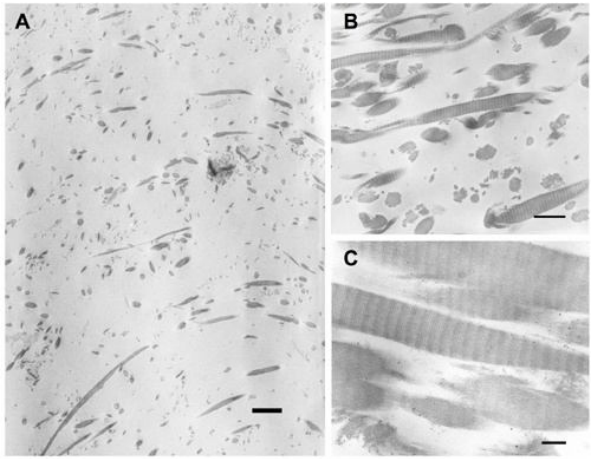

Fig 13 - High resolution images of BIOPAD samples treated for 5 hours at 37 °C with collagenase. Scale. Bars are 2 µm (A), 500 nm (B) and 100 nm (C).

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BIOPAD EQUINE COLLAGEN

As already observed by light microscopy, COLACTIVE® collagen samples collapse into large and densely packed clumps, whenever exposed to collagenase. When these clumps were examined by electron microscopy they appeared as large aggregates of ramdomly dispersed fluffy material. Thus a major effect collagenase exposure induces onto COLACTIVE® samples can be envisioned in the aggregation and collapse of all collagen fibrils, rather than in any alteration of the assembly or periodicity of the single fibrils themselves. It should be recalled here that even control samples of the COLACTIVE® collagen do not exhibit any structural periodicity (see Fig. 7 for comparison).

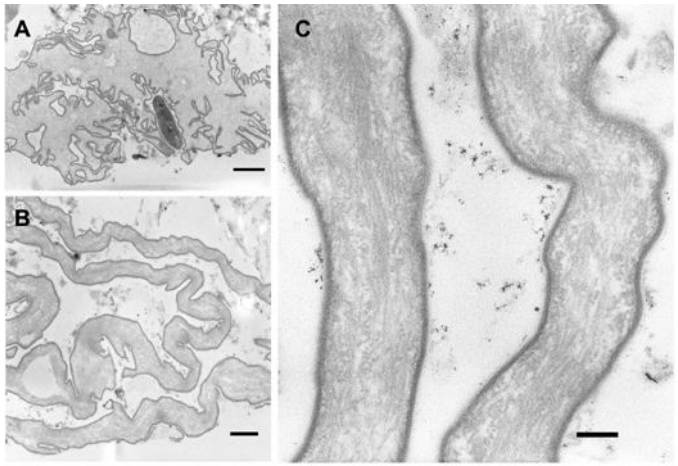

Fig 14 - High resolution images of COLACTIVE samples treated for 5 hours at 37 °C with collagenase. Scale Bars are 1 µm (A), 500 nm (B) and 100 nm (C).

The last sample to be examined by electron microscopy in this study is the PROMOGRAN® collagen. Following exposure to collagenase, this last sample (Fig 15) appeared to retain only large clumps of a densely packed material. When observed by high resolution microscopy, they revealed no periodicity regardless of the ultimate instrumental magnification attained. However, since control samples of the PROMOGRAN® collagen do indeed exhibit typical periodical fibrils (see Fig. 9), it is likely that structurally organized fibrils are easily removed by the collagenase treatment and that only clumps of packed fibrils are retained

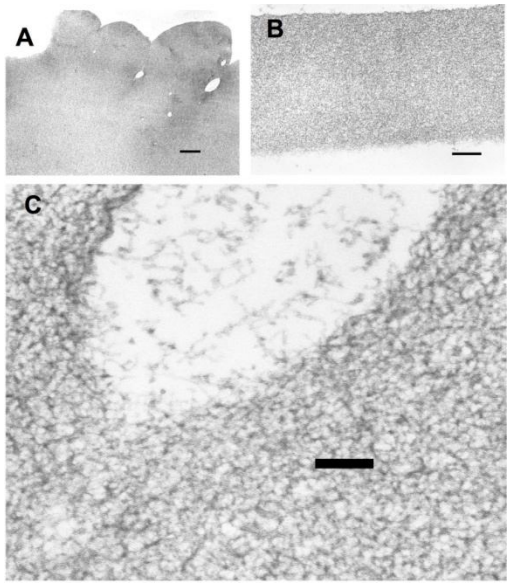

Fig 15 - High resolution images of PROMOGRAN samples treated for 5 hours at 37 °C with collagenase. Scale bars are 2 µm (A), 500 nm (B) and 100 nm (C).

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BIOPAD EQUINE COLLAGEN

To summarize the main observations obtained by electron microscopy on samples treated with collagenase, the following points can be highlighted:

- ***PURACOL***® becomes apparently deprived of thinner threads of the interlaced collagen matrix, but the high density fibrils remain almost unaltered, as due perhaps to their packaging that makes them inaccessible to collagenase
- ***BIOPAD***® retains the same morphological characteristics, but the overall structural appearance of the collagen matrix is much more relaxed. However, single collagen fibres, though highly frayed, maintain their typical alternance of periodical black and white bands.
- ***COLACTIVE***® samples are totally collapsed into compact clumps resolvable as large aggregates of ramdomly dispersed fluffy material by electron microscopy.
- ***PROMOGRAN***® is also retaining only some structurally dense spots, that unlike those in control samples, are devoid of any structural periodicity

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BIOPAD EQUINE COLLAGEN

Cell culturing

Given the evidence provided so far on the structural modification of collagens as induced by collagenase treatment, and considering the actual role these samples would have to play in wound healing, we thought it of interest to examine how they interact with various cell types. For this reason, fibroblasts and human lymphocytes were selected and allowed to interact with collagen substrates for up to 48 or 72 hours. Because fibroblasts play a key role in wound repair by producing such extracellular matrix components as different types of collagens and fibronectin (Clark, 1985), it would certainly be important to verify how these cell types can actually interact with protesic collagen. In particular, it is essential to establish whether they can actually take contact with the three-dimensional collagen matrix and whether they can move and proliferate freely under these conditions (Croce, 2004). On the other hand, leukocytes and lymphocytes have been shown to associate in vitro with collagen gel and to phagocyte fragments of it perhaps as a crucial step in the initiation of the thrombus formation (Gay et al., 1975). At the same time, lymphocytes are known to modulate the production of several growth factors by macrophages so as to stimulate some immune reactions that may eventually lead to connective tissue destruction (Vaes, 1980). In view of these roles, and perhaps for other and more complex reasons, it would be important to determine how lymphocytes migrate in a three-dimensional gel matrix, and whether they simply proceed by pseudopodia extension or by adhesive interactions (Haston et al., 1982). The following figures are meant to illustrate how fibroblasts and lymphocytes do indeed interact with the four collagen samples that are being compared in this study.

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BIOPAD EQUINE COLLAGEN

As it can be clearly seen in Fig. 16, the overall appearance of the collagen matrix does not change very much in the presence of fibroblasts as compared to the controls (see Fig. 1), and thus samples are very different from those depicted after collagenase treatment (see Fig. 11). To find out in more detail how fibroblasts interact with the collagen gel matrix, each of the four collagen samples was examined at the transmission electron microscope.

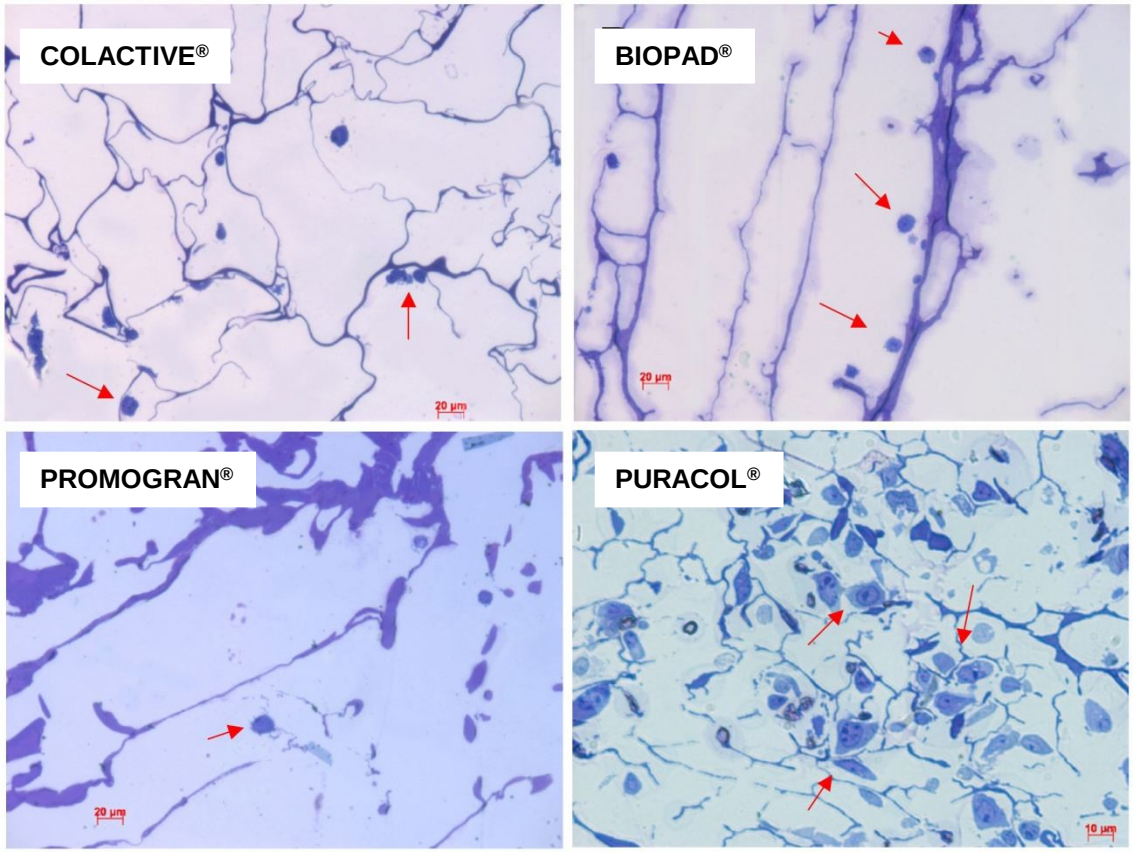

Fig. 16 – Light microscope sections of collagen samples examined after 48 hours of in vitro culture in the presence of 3T3 fibroblasts. Scale of 20 µm are indicated in each picture of COLACTIVE, BIOPAD and PROMOGRAN. Scale bar of PURACOL is 10 µm.

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BIOPAD EQUINE COLLAGEN

Figs. 17 shows a sequence of micrographs of fibroblasts co-cultured in the presence of the COLACTIVE® gel matrix. At low magnification, one can actually see that fibroblasts take firm contact with the surface of collagen fibres. No alteration can be envisioned in the collagen substructure nor pseudopodia can be seen associated with the fibroblast cell periphery (Figs. 17 A and B). At higher magnification, the fibroblast cell membrane shows little contact with the collagen surface and a number of uncoated vesicles that could be either exo- or endocytic in nature. We take these observations as indicating that fibroblasts do not interact very easily, at least within the time period tested in this study, with the COLACTIVE® gel, due perhaps to its superficial gel properties that may make it difficult for the cells to penetrate deeply into the gel matrix.

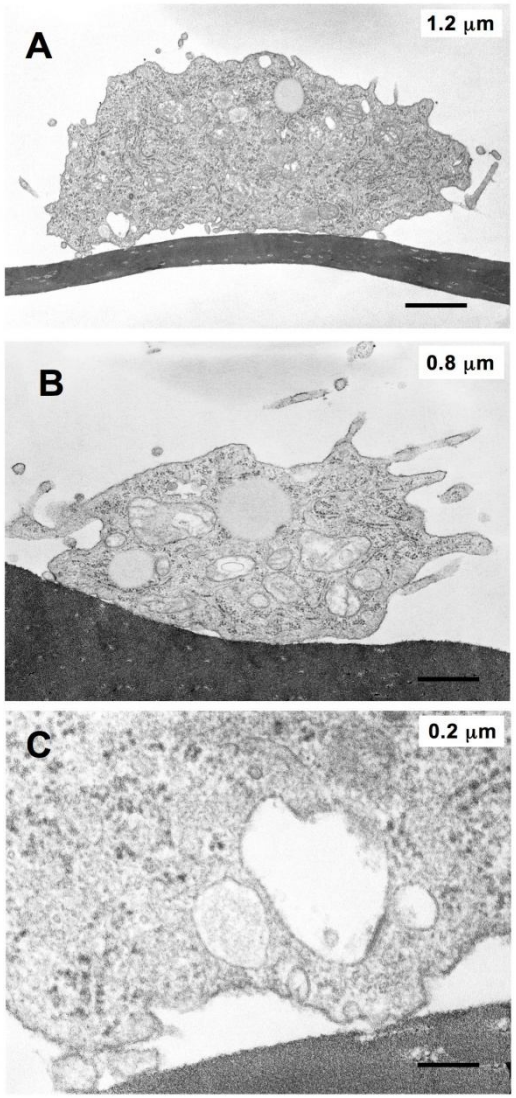

Fig. 17 – Electron microscope micrographs of COLACTIVE gel matrix exposed in vitro for 72 hours to 3T3 fibroblasts

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BIOPAD EQUINE COLLAGEN

Fig. 18 shows how 3T3 fibroblasts interact with BIOPAD collagen when both are placed in co-culture for up to 72 hours. Interestingly enough, these cells become highly intermingled with the collagen matrix, being capable of embracing several frayed collagen threads with a number of extending filo- and lamellipodia (Fig. 18A). Embracement between 3T3 fibroblasts and BIOPAD® collagen may become so tight that several cells may simultaneously be involved in enclosing a cluster of several collagen fibres (Fig. 18B). At higher magnification, one can clearly see how interaction between collagen fibrils and 3T3 cells is actually attained. Fig. 18D shows that this is realized through a direct contact of the cell membrane with a few collagen fibrils that become consequently frayed. Occasionally, collagen fibrils can be seen so close to the cell surface as to give the impression of being internalized by endocytosis. Fig. 18C demonstrates that some electron dense material has gained access to the cytoplasm of a 3T3 fibroblast cell. In addition, the presence of several multivesicular bodies in the proximity of the cortical cytoplasm strengthens the impression that something is being modified intracellularly. Whether this is functionally equivalent to a collagen proteolytic process is taking place cannot be clearly said in the absence of proper markers. At any rate, the observations carried out on the BIOPAD® gel demonstrate that fibroblasts have an active behaviour on this gel matrix and may perhaps contribute to remodel it by either helping to degrade it or to synthesize de-novo some collagen fibrils.

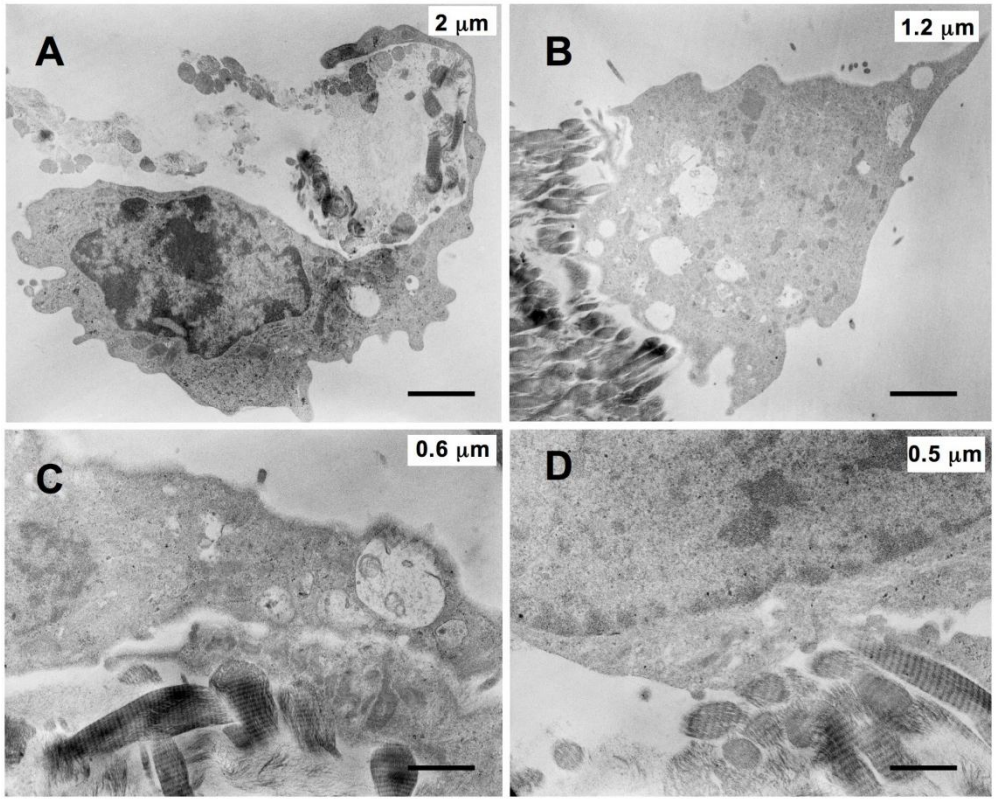

Fig. 18 – Electron microscope micrographs of BIOPAD collagen samples exposed for up to 72 hours at 37 °C to 3T3 fibroblasts in Dulbecco modified culture medium. Scale Bars are 2 μm (A), 1 μm (B), 0.6 μm (C) and 0.5 μm (D).

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When the PROMOGRAN® collagen sample was examined ultrastructurally in the presence of 3T3 fibroblasts (Fig.19), a situation comparable to that already depicted for the COLACTIVE® sample was once again found. Although 3T3 fibroblasts take firm contact with the PROMOGRAN® collagen surface, they never form pseudopodia for locomotion nor they form any membrane infoldings or protrusions indicative of an active interaction. So even in this case the PROMOGRAN® sample appears so densely packed that the fibroblasts are somehow prevented from entering the gel matrix and establishing a more elaborate participation in either degrading the protesic collagen or synthesizing new collagen fibrils.

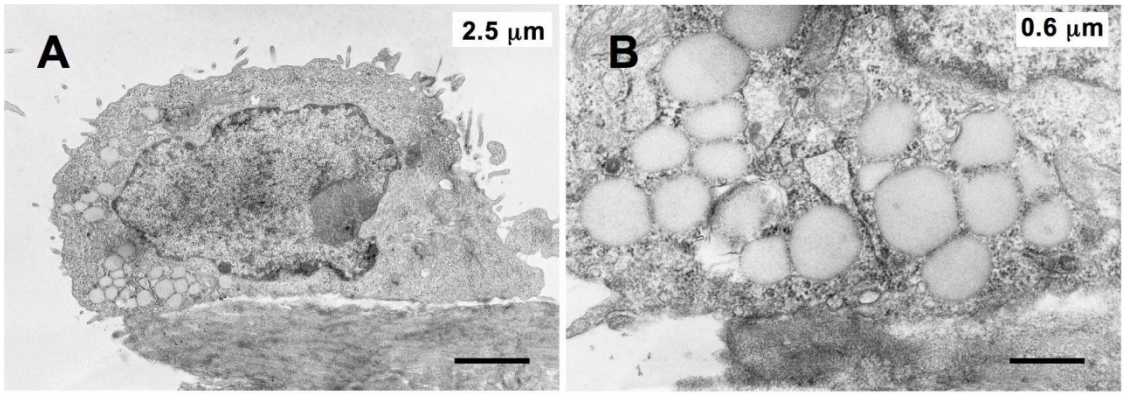

Fig. 19 - Electron microscope micrographs of PROMOGRAN collagen samples exposed for up to 72 hours at 37 °C to 3T3 fibroblasts in Dulbecco modified culture medium. Scale Bars are 2.5 μm (A), and 0.6 μm (B).

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A CHEMICAL, MORPHOLOGICAL AND BIOCHEMICAL COMPARATIVE STUDY OF COLLAGEN WOUND DRESSINGS

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Finally, the last collagen sample examined in this study for testing interaction with fibroblasts is PURACOL®. Fig. 20 shows a sequence of micrographs documenting this interaction. The first one can notice is that collagen fibres are more or less unaltered and in a few points they take contact with the fibroblast surface (Figs. 20A and B). When these contact points are examined at higher magnification, no special feature of the cell plasma membrane can be envisioned. Once again even this sample of PURACOL® appears to react rather passively with fibroblasts, for no sign of any alteration either in the cell or in the collagen itself can actually be found. It remains to be seen whether fibroblasts may be synthesizing some collagen that the micrograph cannot prove.

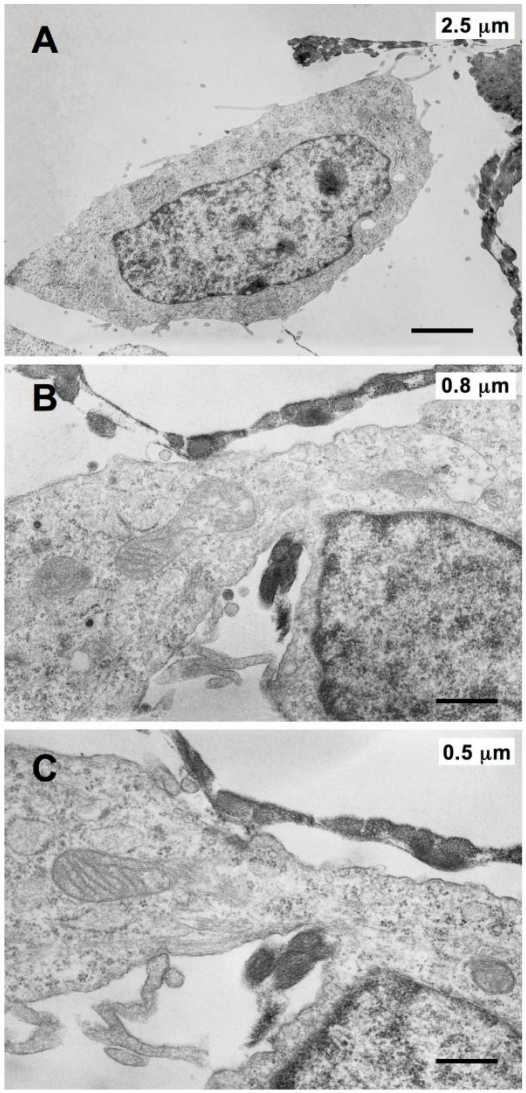

Fig. 20 - Electron microscope micrographs of PURACOL collagen samples exposed for 72 hours at 37 °C to 3T3 fibroblasts. Scale Bars are 2.5 µm (A), and 0.8 µm (B) and 0.5 µm (C)

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White blood cell interaction

To further ascertain the potential role played by these collagen samples in wound healing, we tested their ability to interact with human white blood cells, mainly lymphocytes.

Fig. 21 depicts a series of four light microscope micrographs documenting this interaction. At this low magnification no collagen alteration can be envisioned. So to verify whether blood cells or the collagen matrix itself may actually be structurally modified as a result of this interaction, all samples were examined with higher resolution at the electron microscope.

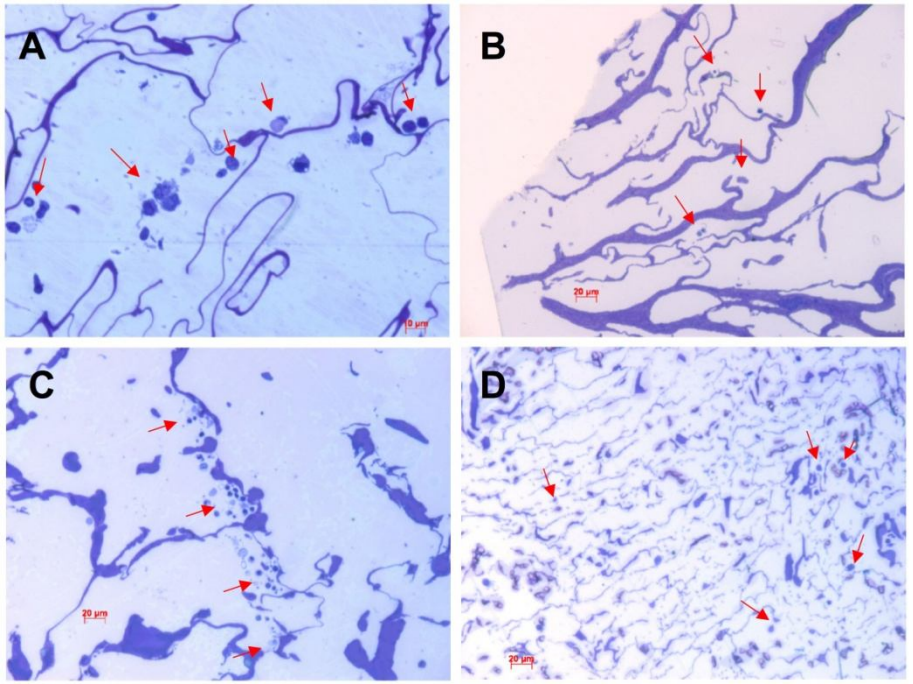

Fig. 21 – Light microscope micrographs of several collagen samples: (A), COLACTIVE; (B), BIOPAD; (C), PROMOGRAN and (D), PURACOL cultured in vitro for up to 72 hours in the presence of human lymphocytes. Red arrows point to the sites where lymphocytes are located. Bar scales are 20 µm for all pictures.

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As it can be clearly seen in Fig. 22, the COLACTIVE® sample can actually undergo several structural modification when exposed to lymphocytes. In particular, one can notice that the periphery of the large clumps of collagen fibrils is disaggregated in the proximity of the lymphocyte surface (Fig. 22A). In addition, the lymphocyte itself is highly modified intracellularly since it exhibits a number of endosomal vesicles, some of which include material structurally similar to the disaggregated collagen fibrils (Fig. 22B). We take this observation as a clear indication that lymphocytes participate actively in the process of wound healing by partially degrading and most likely internalizing all collagen fibrils with which they may come into contact.

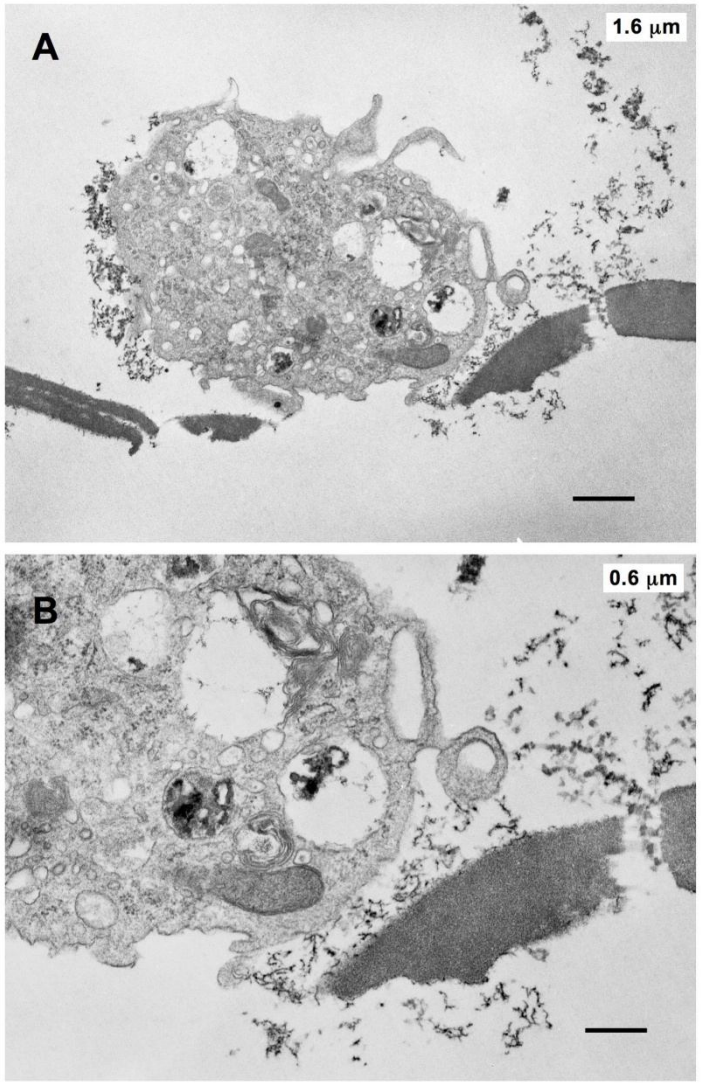

Fig. 22 - Electron microscope micrographs of COLACTIVE collagen samples exposed for 72 hours at 37 °C to human lymphocytes. Scale Bars are 1.6 µm (A) and 0.6 µm (B).

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When the BIOPAD® collagen sample was examined by electron microscopy an even more pronounced pictured emerged, in that lymphocytes appeared more heavily loaded with electron dense material in their endosomal compartment (Figs. 23A and B). In addition, the collagen fibres present in the extracellular milieu appeared highly reduced into clumps of fibrillar material (Fig. 23C). On the whole, these observations demonstrate that the BIOPAD® collagen is highly susceptible of being degraded following interaction and internalization by lymphocytes. This conclusion is based primarily on several structural findings, but they would certainly require more sophisticated techniques to identify the nature of the material being produced and internalized as a result of the collagen-lymphocyte interaction. It is nevertheless highly significative that no such material could ever be evidenced when the BIOPAD® collagen was placed in the presence of collagenase of other cell types as fibroblasts (see Figs. 13 and 18 for comparison)

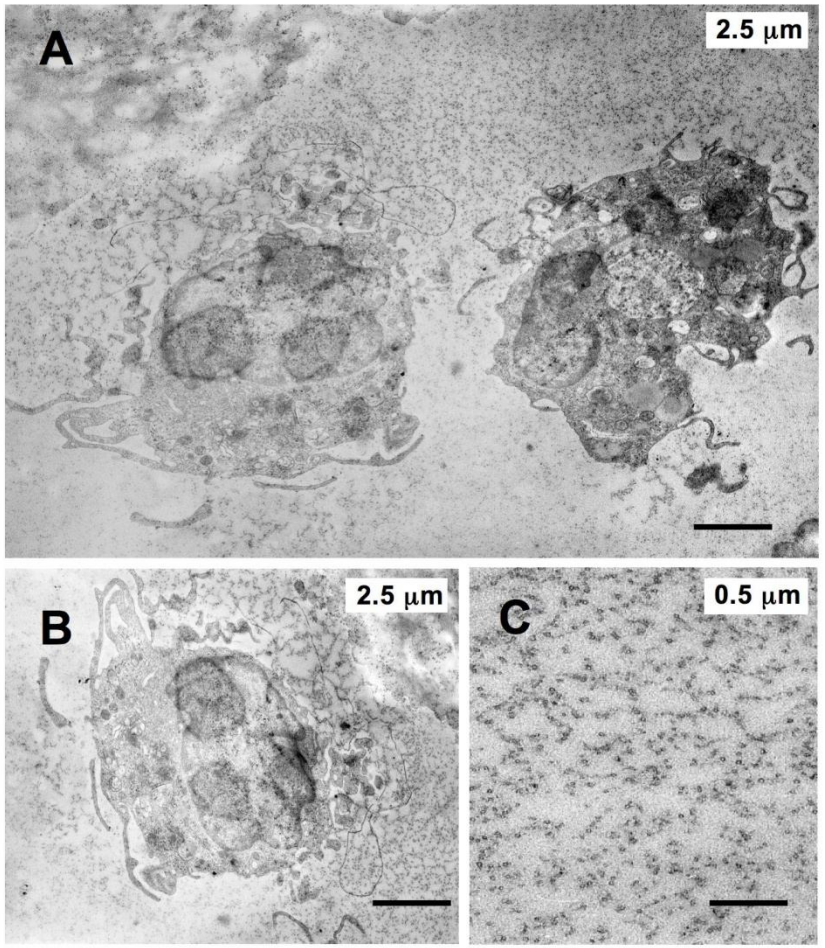

Fig. 23 - Electron microscope micrographs of BIOPAD collagen samples exposed for 72 hours at 37 °C to human lymphocytes. Scale Bars are 2.5 µm (A), 2.5 µm (B) and 0.5 µm (C).

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When this ultrastructural study was extended to the analysis of the PROMOGRAN® collagen sample, we could observe that the gel matrix and the interacting lymphocytes were not at all altered morphologically or only slightly modified in their superficial appearance (Fig. 24A). Although a few plasma membrane indentations can actually be envisioned along the lymphocyte surface, no structural modification can be seen to occur in the gel matrix nor any endosomic vesicle is seen to appear in the lymphocyte cytoplasm (Fig. 24B).

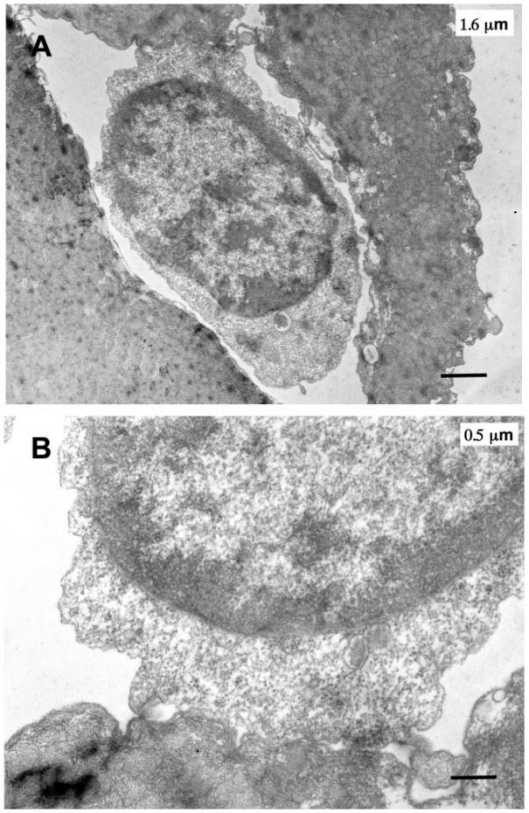

Fig. 24 - Electron microscope micrographs of PROMOGRAN collagen sample exposed for 72 hours at 37 °C to human lymphocytes. Scale Bars are 1.6 µm (A) and 0.5µm (B).

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Finally when the last sample of the PURACOL® collagen in the present series was examined by electron microscopy essentially the same pictures as for the PROMOGRAN® gel could be described. In fact, following interaction with lymphocytes no modification could be recorded in the gel matrix, nor could any change be seen to occur in the lymphocyte cytoplasm. We interpret both these last observations as indicating that PROMOGRAN® and PURACOL® are much more resilient to lymphocytes in that they persist longer unaltered whenever they come into contact. It would be interesting to verify whether this gel property is either enhancing or inhibiting any process leading to synthesis of new collagen fibres by the fibroblasts.

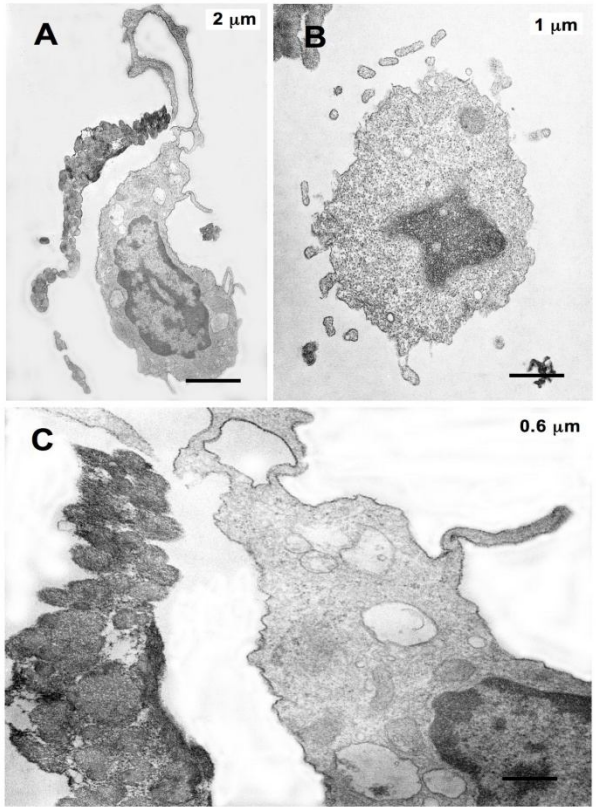

Fig. 25 - Electron microscope micrographs of PURACOL collagen sample exposed for 72 hours at 37 °C to human lymphocytes. Scale Bars are 2 µm (A), 1 µm (B) and 0.6 µm (C).

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CONCLUSION

The application of several morphological and biochemical techniques to the study of four different collagen samples allowed us to draw the following conclusions. All samples are characterized by an interlaced matrix bordered by collagen strands. The parameters to be considered are the actual dimension of the mesh substructure and the thickness of the collagen strands. Meshes too large or to small may not be suitable for sustaining any cell movement during the healing process. At the same time, collagen strands that may be either too thin or too thick may compromise the actual persistence of the medical device on the wounded area, due to a low collagen content per unit volume. In our view, the BIOPAD® sample exhibits the best structural compromise between the extension of the mesh areas and the thickness of the collagen strand. This conclusion is also corroborated by the analysis carried out at the scanning electron microscope where only the collagen strands of the BIOPAD® could be seen as made of thick laminas of packed fibres. At this level, all other samples had either strands that are too or fibre bundles that are too highly and densely packed.

When examined at higher resolution all sample, except COLACTIVE®, revealed the presence of a staggered pattern of black and white bands. However, even at this level of analysis one could clearly see how collagen fibrils are packed together to make up the collagen strands of the interlaced matrix. Too many fibrils make the collagen strands too thick as in the case of PROMOGRAN®. At the opposite end, fewer fibrils make the collagen strands too thin and thus too fragile to persist during the healing process. The electrophoretic analysis reveals that all samples are comprised of pepsin-liberated polypeptides with molecular weights identical to those of the type I collagen. These structural and morphological characteristics can be properly evaluated when all collagen samples are assayed in relation to cell invasion during the healing process. Accordingly such cell types as fibroblasts and lymphocytes were selected and co-cultured in vitro in the presence of all collagen samples. The criteria by which their interaction was evaluated were focused on the structural modification induced in the gel matrix and such cell parameters as membrane infoldings and appearance of endosomal vesicles. As far as gel matrix modification is concerned only COLACTIVE® and BIOPAD® appear to be sufficiently well altered when placed in contact with lymphocytes, thus suggesting that they may be easily accessible during wound healing and rapidly degraded. However, of these two samples only the BIOPAD® appears to be involved in a more active interaction with the fibroblasts, as signified by the appearance of several membrane indentations along their cell contour. Other samples differ both in their capacity to degrade protesic collagen and to sustain any active interaction with the fibroblasts.

Taken together the evidence provided in this study documents quite clearly how different collagen medical devices can actually interact with the enzymes and the cells that characterize the environment of a healing wound. Since collagen is known to perform a major role in restoring strength and remodelling scar tissue (Madden and Peacock, 1971), it is to be expected that such enzymes as collagenase and such cells as fibroblasts may actually modify the structural appearance of the exogenous collagen. And depending on the way these factors interact with collagen, the kinetics of wound healing will be consequently affected. Unlike all other collagen samples tested in this study, BIOPAD® proved to be the only one capable of retaining the same overall molecular structure during exposure to collagenase, and, at the same time to allow the collagen interlaced matrix to be clearly frayed off. This is undoubtedly one of the best conditions for sustaining specific interactions with all cells deputed to wound healing. As we have documented in this study, BIOPAD® frayed collagen fibrils can in fact be seen close to the cell surface or even be endocytosed by lymphocytes, suggesting that this may be a favourable condition for would healing and remodelling.

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