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Orthopaedic Hospital and University of Southern California

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OPTICAL BONE CHAMBERS AS TOOLS FOR STUDYING THE BONE-IMPLANT INTERFACE: A REVIEW

Howard Winet

Orthopaedic Hospital and University of Southern California, Los Angeles, CA

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Abstract

Bone chamber intravital microscopy combines the control volume of in vitro models and the chemical complexity of in vivo models to optimize the study of bone physiology in situ. As an optical tool it provides a window to dynamic events at the tissue level of magnification. In particular, it allows measures of microvascular events: (1) in space by magnifying local perfused vasculature and microcirculation at any instant, and (2) in time by providing the same volume of tissue for weekly viewing of an evolving process such as bone healing. This quartz-bearing titanium screw has revealed a consistent order for vascular-bone progression during healing. Videography and digital image processing allowed simultaneous measurement of osteogenesis, angiogenesis, blood supply and permeability.

Key words: Angiogenesis, bio-erodible implants, intravital microscopy, optical bone chamber, osteogenesis, polyglycolide, polylactide, vascularity.

Impact of microvasculature on the healing process

Bone shares with other tissues a requirement to proceed through haemostasis, inflammation, clearance and repair in order to close a wound. Unlike nearly all the others, however, modeling includes regeneration and must be followed by remodeling if the tissue is to regain its original ability to bear mechanical stress. Accordingly, bone must heal even microfractures to avoid the fatigue-failure common to other somewhat rigid nonliving composite materials.

The Bone Chamber Laboratory’s (BCL) interest has been the role of microvasculature in fracture/defect healing. While many components of fracture healing physiology can be studied in vitro, e.g., osteoblasts may be grown under various oxygen tensions, their coordination in the healing process is not a simple sum of component transactions. Accordingly, in vivo models are necessary. But, they must be kept simple enough to keep track of each parameter. This goal would seem to be inconsistent with using intact subjects. But, while it may not be possible to reach the "purity" level of in vitro models, one can still achieve an optimal balance between the accessible reproducibility of in vitro measures and the elusive reality of "normal" in vivo measures by developing an intermediate model which retains salient features of each venue.

Conventional in vivo studies of vascular physiology during fracture healing have not been intermediates. They have been global and either acute or chronic. Acute studies obtain measurements at one time but are not necessarily restricted to a single measure. They all, however, share a need to collect the tissue to be measured; as when McGrory et al. (1994) obtained at least three blood flow measurements over a four hour period in single subjects using entrappable isotopic microspheres.

Black box and histological measures of osseous microvasculature have been useful for answering limited questions about bone microvascular physiology. A more direct link between structure and function will have to be
established, however, before physiological mechanisms for such conditions as atraumatic ischemic osteonecrosis can be ascertained.

Bone chamber implants (BCIs), a chronic local sampling technique

The need for direct local measures motivated Sandison (1924) over seventy years ago to develop a window for chronic observation of microvasculature in situ. His rabbit ear chamber has been used extensively for studying wound healing, particularly by T.K. Hunt and his colleagues (Hunt and Goodson, 1988). The device is essentially two coverslips riveted into a rabbit’s ear, covering both ends of a punch-hole. Ear tissue heals into the fluid-filled (healing will not occur into air) space between the slips forming a sandwich of about 50 μm which is viewed through a microscope. The ear chamber is well-tolerated by supporting tissue and can be left in place for chronic studies.

Moreover, relatively short working distances are possible so high numerical aperture objectives may be used, allowing for sufficient magnification to resolve individual cells. Hunt and his coworkers have used this tool to study, among other things, the interplay of oxygen, endothelial cells, macrophages and pH during wound repair (Knighton et al., 1983). One of their findings which bears on the BCL’s current work is that lactic acid stimulates macrophages to secrete angiogenins.

Chronic studies on internal organs using window implants have been limited more by the opacity of the structure than its tolerance of the implant. Accordingly, investigators have been limited to windows which expose organ surfaces and utilize epi-illumination for organs like liver (McCuskey, 1986) and brain (Pawlick et al., 1981). Mesentery, in contrast, is not opaque but must be studied ex vivo and, therefore, acutely.

Since Wieder’s (1907) bone defect model was developed, a control volume technique for studying healing has been available to histologists. However, physiologists wishing to view fracture healing in bone had to reconstruct details from indirect measures and histology. Although, Kirby-Smith (1933) applied the ear chamber to study an accidental fracture in transplanted bone, window implants for bone have been developed almost exclusively by Sandison’s Swedish neighbor Per-Ingvarg Bränemark and his colleagues (Bränemark, 1958, 1959; Bränemark et al., 1964), in particular, Tomas Albrektsson (Albrektsson, 1980a, 1980b, 1980c, 1980d, 1981, 1983, 1984, 1987; Albrektsson and Albrektsson, 1978; Albrektsson et al., 1980; Albrektsson and Linder, 1984). Their names are familiar to dentists and maxillofacial surgeons because of one of the clinical consequences of their window implant research, the Bränemark oral prosthesis. Bränemark’s original interest was microcirculatory aspects of hematopoiesis. His observation of the reversible deformation of red blood cells in narrow vessels (Bränemark, 1959) is a classic paper in haemo-rheology and his demonstration that blood flow in bone is not sluggish helped demystify bone physiology by making it more like that of other organs, thereby fulfilling the scientific requirement for parsimony.

He accomplished these observations before developing a window implant. In 1958, he ground down the cortex of a rabbit fibula until little more than endosteum remained (Bränemark, 1958). By bringing a light beam through a hole in the opposite cortex, Bränemark was able to illuminate the endosteal and nearby medullary vessels. These observations were acute, however, and in order to carry on chronic observations, he developed an implant based upon bone screw technology which was already being used for fracture fixation. While the optics of window development were a challenge, the most decisive advance in establishing the total implant as a biocompatible chronic device was achieving osseointegration of screw threads with host bone. Bränemark realized that the same effect was required for success in the design of implants for edentulousness and the result has been a revolution in maxillofacial reconstruction.

The experimental device invented by Bränemark et al. (1964) is the optical bone chamber (BCI). It consisted of a hollowed pure titanium cylinder which was threaded on the outside and filled with two quartz windows. The head quartz rod, in the part of the bone chamber which protruded from the medial proximal surface of a rabbit tibia, was the shorter of the two. It was separated by about 150 μm from the 2 x 30 mm tail rod, contained in the end of the chamber which protruded from the opposite or lateral surface. Healing tissue would grow into this separation or "slit" and be observed using trans-illumination through an intravital microscope (IVM) which could accommodate the rabbit on its stage. The type of tissue which regenerated into the slit was a function of its radial location. If it was central to the endosteum, the tissue would be marrow. If sufficiently radial, it would be cortical bone.

Albrektsson refined the chamber for easier implantation and management in the 1970s, as did McCuskey and his colleagues (McCuskey et al. 1971; McClugage and McCuskey, 1973; Rosenblum et al., 1976). This latter group, at present the only group outside of Sweden to apply the optical BCI, used one-piece molded lucite chambers in place of titanium and quartz. The McCuskey group concentrated on investigating hematopoietic agents. Albrektsson, however, was more interested in the osseous component. He was the first to study incorporation of autogeneic bone transplants, finding that:

1. Autogeneic lamellar bone transplants join with
Bone chambers for implant study

recipient host bone and receive host vasculature without forming an interspace unless high-speed cutting tools are used to prepare the recipient site (Albrektsson, 1980a).

2. Autogeneic lamellar bone transplants are vascularized within 5 days of implantation (Albrektsson and Albrektsson, 1978).

3. Cutting cones penetrate autogeneic lamellar bone transplants at a speed of 30-40 μm/day (Albrektsson, 1980b).

4. Necrosis of bone around curing polymethylmethacrylate penetrates to a depth of about 200 μm in cortical bone and healing required no more time than bone killed by other methods. Marrow, however, showed a much slower than normal recovery with the greatest damage noted in its vasculature (Albrektsson and Linder, 1984).

5. Fifteen hundred rads of ionizing radiation is sufficient to slow the onset of remodelling in healing cortical bone (Albrektsson et al., 1980).

6. Artificially perfused suspensions do not fill more than 40% of targeted microvessels (Albrektsson, 1981, 1984).

7. Host tissue vessels in healing cortical bone anastomose spontaneously during autogeneic bone incorporation (Albrektsson, 1980c).

8. Bone blood flow is retarded after a "few" hours of pulsed electromagnetic field (PEMF) stimulation at 15 Hz (Nannmark et al., 1985).

9. Direct current stimulation up to 20 μA stimulated angiogenesis, but values closer to 50 μA caused vessel resorption (Buch et al., 1986).

10. Mechanically stressing a chamber before osseointegration will retard angiogenesis (Albrektsson, 1983).

Recently, an optical plate bone chamber was reported by Boyde et al. (1995). This device allows resolution of cells similar to that achieved by Albrektsson with his short head BCI (Albrektsson, 1987). The Boyde device is more like a half-ear chamber; with a single window covering a bone defect which has direct communication with the medullary canal. Moreover, it utilizes confocal intravital microscopy in place of bright-field illumination used in our chamber. Thus, it can perform optical sectioning better than previous bone windows.

Thus, the BCI window is a device uniquely suited to studying in vivo events as they occur and over long periods of time. As applied by its inventors, it revealed qualitatively detectable responses to stresses electrical, thermal and mechanical.

Chronic Viewing of Bone Healing Through a Window

During the past 10 years the BCL has attempted to convert the BCI to a quantitative tool so that its visual data could be used to test hypotheses statistically. It was reasoned that by measuring all visible vasculature, bone, and transport at each observation, one could periodically obtain sufficient physiological data to detect cause-and-effect time-dependent relationships. In addition, it seemed reasonable that weekly observations would allow detection of changes in angiogenic and osteogenic patterns. This change in approach meant that the ends of the implant would have to remain exposed throughout the observation period. Finally, in an effort to avoid anaesthetic effects on blood flow, the intravital microscope was redesigned to allow viewing of an awake rabbit while placing no pressure on tissue compartments surrounding the BCI. Accordingly, a horizontal intravital microscope was constructed and the recording medium was switched from ciné to video. A sketch summarizing its essentials is presented in Figure 1.

The amount of microvasculature in a tissue disk 100 μm thick and 2 mm in diameter can be considerable. Accordingly, digital image processing was necessary for extraction of data for analysis. Frame grabbers were employed to process images and software developed to
Figure 2. Vascularity and bone volume in the slit-gap compartment during defect healing from W3 through W9. • = bone fraction; ■ = vascularity. It must be remembered that only perfused vessels are evident in this model. Vascularity peak during inflammatory hyperemia occurs at about D10-W2 so is not detected during first observation. Decrease in L/V after second peak appears to indicate a return to steady state remodeling typical logistic growth curve.

The BCL BCI

The first task was to develop a control model which would provide baseline data for subsequent projects. Instead of the transplant which characterized Albrektsson's model, the control BCL BCI carried only injected blood from the implanted rabbit's medullary canal. Thus, the control was a defect-healing model which accommodated porous ingrowth. That is, cortical bone would appose into the slits the 100 μm window separation and across a cell/gap initially occupied with host blood/hematoma. The disk-shaped visible volume was termed the "slit-gap" compartment.

Viewing of the slit-gap begins at surgery when medullary blood from the burred defect is injected into the compartment. The resulting clot traps mesenchymal stem cells in a fibrin matrix. At this time, dispersal of a blood film across the slit-gap is the only visible event.

No vessels have penetrated the slit-gap by the end of W1 (first week post-implantation). They must come from Haversian canals through about 1 mm of necrotic bone and 1 mm of hematoma outside the compartment. By W2, there is circumstantial evidence that macrophages have penetrated the necrotic bone ring as the hematoma is no longer visible and the slit-gap appears transparent. Vessels may be present but observations have varied considerably. They are often convoluted, showing no preferred orientation in accordance with Postacchini's et al. (1995) observations of early bone defect healing.

Between W2 and W3, vascular invasion is substantial, a pattern reminiscent to that of vascular invasion by 15 days in human periosteum over closed diaphyseal fractures (Postacchini et al., 1995). This wave of angiogenesis has been referred to as an inflammatory event. However, it is not the standard wound healing reaction which is completed by D10 (10 days post-implantation). Since the slit-gap is sealed off from vascularized tissue and can only be supplied through initially necrotic bone, vessels cannot penetrate until clearance is completed in the slit-gap.

At W3, the ends of the BCI are exposed, and skin around the wound permanently reflected with screw-on buttons. Now osseointegrated, the titanium threads assure that mechanical stress to the slit-gap tissue will be limited to its impending connection with cortical bone facing the slits. Vascularity, expressed as total vessel length per unit tissue volume (L/V in μm/cm²), is extensive. Bone is extremely rare at this time although temporary primitive woven bone islets like those reported in Albrektsson's (1987) BCI s have appeared. The inconsistency of this finding casts some doubt on the contention (Shapiro, 1988) that woven bone appears in mechanically stable gaps > 0.5 mm. Histological studies by Albrektsson (1987) have identified the tissue at this time as fibrovascular.

Measures of defect healing in controls

Measurements of vascularity and bone (fraction of compartment filled) from W1 through W9 are presented in Figure 2. The curve slopes are, of course, neoangiogenesis and neoosteogenesis respectively. Corresponding clinical terms would be angiogenesis and bone formation rate. Measurements were obtained by tracing video frame-grabbed images with various image analysis computer programs and processing the digitized values.

Bone appears at the edge of the compartment at W4 when vascularity is lowest. From the standard assumption that vascularity is directly correlated with partial pressure of oxygen (PO₂), one may conclude that these results agree with Brighton et al.'s (1991) finding that osteoblast proliferation is favored at low PO₂. By the same token, the higher vascularity at W3 agrees with their other finding that high PO₂ favors macromolecular (implying collagen) synthesis.

Since the entrance of bone into the slit-gap mimics porous ingrowth of trabeculae, BAR (bone apposition rate) is a logical representation of BCI osteogenesis in standard dimensions. As suggested by the plot slope, ossification is faster than after W8 in both the BCI and standard models. While BAR is effective as an
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Figure 3. Blood supply and bone volume in the slit-gap compartment during defect healing from W3 through W9. • = bone fraction, ■ = blood supply. Q represents a maximum value since it is measured over a ≥ 2 minute period and microspheres do not appear in all vessels simultaneously. As might be expected, given that L/V represents perfused vasculature, the relationship Q versus time is similar to that of L/V versus time.

indicator of osteoid formation, increase in bone volume as an indicator of net mineralization may be measured in the bone chamber as \( \mu m^3 \) of bone per cm\(^3\) of total tissue (given the slit-gap compartment as a control volume) per day as indicated in data summarized in Table 1. Remodeling is also detectable in the BCI, although it has not yet been quantified. Haversian canals do not remain the same from week to week and parts of ingrowing trabeculae seen at one observation are missing a week later. Resorbed trabeculae would not be detectible in a model which sampled each rabbit once only.

Angiogenic rates in bone do not appear to have been reported beyond the BCI data of Albrektsson (1987) presented in Table 1. Average rate of growth for a single vessel may be a useful measure for simple comparisons, but the rate of vascularization of a given volume of tissue contains far more physiologically relevant information. Accordingly, \( \mu m \) of vasculature per cm\(^3\) of total tissue per day (L/V per t) is used for BCI results. From Figure 3, it is evident that vessel resorption precedes the appearance of bone and may appear again as lamellar bone (Albrektsson, 1987) with its secondary osteons become commonplace late in the healing process. "May" is used in the previous sentence because only one control animal has undergone vessel measurements this late.

Where do the vessels grow while the fibrovascular matrix ossifies? One can readily see by optical sectioning that they are in the ossified tissue (Winet, 1989). The vascularity baseline at W4 in Figure 3 is a fibrovascular condition. As a rule, the increase in L/V results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Post-Trauma</th>
<th>Conventional</th>
<th>Bone Chamber Implant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Apposition Rate</td>
<td>W4 &lt; 110 ( \mu m/day ) (Galante et al., 1971)</td>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td>W4 ——</td>
<td>73 ( \mu m/day ) same</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td>W4 &gt; 55 ( \mu m/day ) (Galante et al., 1971)</td>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td>W8 ——</td>
<td>21 ( \mu m/day ) same</td>
<td>——</td>
</tr>
<tr>
<td></td>
<td>General 6-15 ( \mu m/day ) (Schenk, 1987)</td>
<td>——</td>
<td>( 2.4 \times 10^6 ) ( \mu m/cm^3/day )</td>
</tr>
<tr>
<td>Angiogenic Rate</td>
<td>Maximum 300 ( \mu m/day ) (Albrektsson, 1987)</td>
<td>158 ( \mu m/day )</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.2 ( \times 10^6 ) ( \mu m/cm^3/day )</td>
<td></td>
</tr>
<tr>
<td>Blood Supply in cm(^3)/min/100gm</td>
<td>W4 &lt; 25 (Nutton et al., 1985)</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W5 11 (Nutton et al., 1985)</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W7 5 (Paradis and Kelly, 1975)</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W8 6.5 (Paradis and Kelly, 1975)</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>General 4.3 (Li et al., 1989)</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>Vascularity W3 4 vessels/1cm (^2) projected (Kusiak et al., 1985)</td>
<td>0.036</td>
<td>( 7.78 \times 10^7 ) ( \mu m/cm^3 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>W4 0.02 (Ganey et al., 1992) -0.014gm (fract. vs contr. tibia) (Wray and Lynch, 1959)</td>
<td>0.033</td>
<td>( 6.79 \times 10^7 ) ( \mu m/cm^3 )</td>
</tr>
<tr>
<td></td>
<td>W8 ——</td>
<td>0.039</td>
<td>1.24 ( \times 10^8 ) ( \mu m/cm^3 )</td>
</tr>
<tr>
<td>General 0.062 (Haansen, 1993)</td>
<td>0.040</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

because apposing trabeculae are more vascularized than the tissue they replace.
Blood supply follows a similar pattern as shown in Figure 3, primarily because LIV represents only perfused vessels in this model. A consequence of this restriction is a relationship between blood supply (Q) and LIV which, in the control, was found to be (Winet, 1994):

\[ Q = 16.4 \left( \frac{LIV}{V} \right) - 23.0. \]

Since this is an empirical relationship, its physical meaning may not become evident until a comparison can be made with non-controls.

In order to make L/V and Q values relevant to established fracture healing models, BCI measures had to be compared with those obtained by conventional techniques. Four variables, considered central to the healing process, were chosen: (1) BAR, (2) angiogenesis, (3) blood supply (Q), and (4) vascularity. A comparison of these measures is presented in Table 1.

In general, the BAR tendency to decrease with time in the BCI agrees with conventional data. One can even place the W4 and W8 BCI values in the "Conventional" column without disturbing the pattern. The measure \( \frac{mm^3}{cm^2/day} \) would seem, however, a more accurate measure of general metabolic activity for the entire tissue.

Angiogenesis is actually being compared between bone chambers, so the term "conventional" is not representative. Unfortunately, no qualified value could be found. Why such a difference in the same model? Albrektsson (1987) measured individual vessels while total vessel length was measured in the present model. Selection of one vessel can hardly be considered representative. Accordingly, \( \frac{mm}{cm^3/day} \) appears a more useful measure of vascularization of the entire tissue.

It is in measures of blood supply that differences between local and global bone models become most apparent. Not only are the values at least four times higher in the BCI, but the tendency for Q to decrease after W3 remains constant in the global model while it reverses at least once in the BCI. That this comparison is between rabbits and dogs is not an adequate explanation. Accordingly, the result has been explained as being partially due to the purity of healing tissue in the slit-gap compartment as compared with the entire callus in the conventional model (Winet and Bao, 1990).

The primary function of circulation in any organ is nutrient exchange. Bone physiology cannot be understood without an account of how and when materials enter and depart the interstitial matrix.

Although the two-pore model for vessel permeability is undergoing major changes, one can distinguish a permeability for albumin-sized molecules different from that for myoglobin-sized molecules. As a first measure of nutrient exchange, permeability to the larger molecule, the incidence of large pores (called "leak points per unit volume of tissue (LP/V)") was evaluated in the BCI during healing and it was found that the greater the rate of osteogenesis, the greater the incidence of large pores (Winet and Bao, 1991).

These measures of defect healing established the control model which is the standard for interpreting effect of perturbations such as the addition of implant materials to the slit-gap compartment.

Thus, the optical BCI appears to yield measures which are reasonable extensions of conventional models with two notable differences: (1) blood supply values are significantly higher due to the homogeneous nature of the tissue sampled, and (2) a number of measures have not been obtained from conventional models (e.g., angiogenesis).

**Chronic Viewing of Erodible Implant "Incorporation"**

**Poly(α-hydroxy acid) (PAHA) implants**

Erodible polymers are becoming increasingly attractive as modalities for bone fixation and reconstructive surgery. Two α-hydroxy acids, lactic acid and glycolic acid are the most utilized monomers. They are polymerized into PAHAs. Like other erodible polymers, they offer the advantages of eliminating revision surgery for removal of metal, and of delivery of osteoinductive molecules. In the rush to develop the ideal device, however, technology has outdistanced its basic science foundation. For example, 8% of polyactic acid (PLA)-plated ankle fractures have been beset by aseptic foreign-body reactions after 12 weeks of implantation (Böstman et al., 1990). Acidosis has been postulated as the probable mechanism (Daniels et al., 1992), and the lactic acid monomer generated by its hydrolysis cited as the agent. Neither in vitro (Li et al., 1990a,b,c) nor in vivo (Vasenius et al., 1992) studies have confirmed this conclusion. Yet, so little of the basic science of the PLA erosion process is known that a reasonable hypothesis for an alternative mechanism has been elusive.

Investigations of polymer erosion usually include two approaches: (1) in vitro studies in which a baseline erosion pattern is determined under well-defined condi-
tions, and (2) in vivo studies for documenting the partic-
lar effects of the host tissue bed. For bone, in vivo
studies are typically indirect. Histology is performed on
harvested polymer samples from sacrificed host animals
to determine tissue reactions. Unfortunately, embedding
and staining solutions tend to dissolve PAHAs. Conse-
quently, it is difficult to histologically assess relation-
ships of polymer and tissue bed. Polarized light micros-
copy is a promising tool for locating birefringent erosion
remnants (Böstman et al., 1992), and one can alternate
stained and unstained sections to reconstruct a snapshot
of the implant environment. Nevertheless, harvesting
techniques miss the interaction of eroding polymer with
its surroundings, the fluid matrix and cells of host tissue.
Direct observation of these events in situ in an intact liv-
ing animal will help overcome this problem.

Of the synthetic polymers currently available for
human implantation, the PAHAs PLA and polyglycolic
acid (PGA) have the longest clinical history. PLA has
been used as a surgical implant material for at least 25
years (Böstman, 1991). It depolymerizes to form pyru-
vic and lactic acid which are eventually metabolized to
CO₂ and H₂O if sufficient O₂ is present. PGA depoly-
merizes to form glycolic acid which may be excreted or
transformed to pyruvic acid. The reported molecular
weights (form of M not always specified) of the implan-
ted devices ranges from 40 to 100 kDa.

PAHAs depolymerize via hydrolytic scission which
is accelerated in vivo by increasing pH (Chu, 1985).
The rate of depolymerization varies with the molecular
configuration of the polymer. In general, PLA, with a
half-life of 195 days, degrades slower than PGA, with
a half-life of 90 days (Chu, 1985). But, the latter has a
greater tensile strength (Hollinger and Battistone, 1986).
There are other sources of polyester implant variation,
such as, crystalline-to-amorphous ratio which relate to
isomer ratios. As an example of the latter, poly(L-lactic
acid) is more crystalline and resistant to hydrolysis than
poly(DL-lactic acid) which is amorphous (Daniels et al.,

At present, it is not possible to control the structure
of a given batch of DL homopolymer so that the se-
quence of racemes is known (Lane and Sandhu, 1987).
Thus, erosion rate of two batches of the "same" polymer
may not be the same in identical environments (Hollin-
ger and Battistone, 1986) and data from well-controlled
in vitro tests show significant dispersion (Lewis, 1990).
Such variation in polymer structure, independent of
molecular weight, must be considered when interpreting
observations.

Orthopaedic application of erodible polymers for
internal fixation has been explored for over 10 years
(Finnegan, 1989). Notwithstanding the challenges to
synthesis reproducibility, attempts to combine the me-
chanical advantages of PGA with the erosion advantages
of PLA, have led to the development of PLGs, polylace-
tide-polyglycolide copolymers of PLA and PGA. Com-
mon, commercial PLG formulations have molar ratios of
50:50 and 85:15 (PLA:PGA), with the former preferred
for maxillofacial bone reconstruction (Hollinger and
Battistone, 1986). The half-life for dissolution of 50:50
PLG can be as rapid as 7 days, but a shift of the molar
ratio in either direction increases this value (Hollinger
and Battistone, 1986). Fixation with PLG has been
reviewed by Böstman et al. (1987), and maxillofacial
grafting by Hollinger and Battistone (1986). Johnson et
al. (1988) loaded PLG strips with human BMP and ap-
plicated them to femoral non-unions for up to 6 months
with no reported foreign-body reactions. However,
Böstman et al. (1991) found that 5% of ankle fractures
fixed with rods and sutures made of 90:10 PLG develop-
sterile draining sinuses after 12-16 weeks and 7% de-
veloped malunions, the latter suggesting mechanical fail-
ure. In response to this result, these workers developed
PGA fiber-reinforced PGA plates and screws. While
mechanical results appeared to be promising, the ten-
dency for both PGA and PLA fixators to stimulate a late
(12 weeks to almost 3 years) foreign-body reaction in
almost 8% of the cases (Böstman, 1991), is cause for
concern.

In a majority of the applications by the Böstman
group, the polymer was essentially a composite, even
though its component monomers were the same species.
This synthetic anisotropy and the natural tendency for
isomer and form (crystalline versus amorphous) variabil-
ity within any polymer batch makes it difficult to main-
tain precision for comparison studies. Intermediate
breakdown products will retain these heterogeneities to
varying degrees. Sufficiently basic information about
interactions between breakdown products with recipient
host tissue should be applicable to both fixation and
grafting. Indeed, given that erodible polymeric screws
must be replaced by bone and that eroding plates may
form part of a callus, one may view fixation with erodi-
ble implants as a form of grafting. There are limitations
to such extrapolation, of course. Also, to the extent that
membranous and endochondral bone healing differ, care
should be taken in predicting membranous maxillofacial
healing from callus-forming long bone models.

The shape of a polymer implant acting as an osteo-
conductive support matrix (OSM) has an important influ-
ence on both its physiologic effects and mechanical sta-
bility. The rate of water imbibition is strongly related
to OSM surface-to-volume ratio (S:V) and surface con-
tour. As the surface area of the OSM increases relative
to its total volume, more surface is exposed to the inter-
stitial fluid, thereby enhancing imbibition. However,
because of the complex diffusion and shell permeability
relationships of bulk erosion, and the fact that these relationships change with device volume (all other things being equal), size alone will not predict degradation rates (Li et al., 1990a). Surface-to-density ratio (S:ρ), however, is inversely related to imbibition and hydrolysis (Törnäälä et al., 1991). Törnäälä et al. (1991) found for PGA fiber-reinforced PGA rod implants, a strong correlation between S:ρ and retention of high elastic modulus with time.

The initial tissue response to a PLG OSM is probably due to mechanical perturbation of the host tissue bed. Because PLG is relatively biocompatible, the few reports of early foreign-body reactions describe a mild transient response (Hollinger, 1983). The late appearance of foreign-body reactions suggests a reaction to polymer remnants which have not been hydrolyzed. If these remnants are particles which act in a manner similar to polyethylene particles, tissue reaction would be closely linked to macrophage activity.

The PLG-OSM reaction to initial tissue edema is probably also mechanical, due to changing pressure environment (osmotic and hydrostatic). Chu (1985) has postulated that in vivo, the amorphous regions of PLG thread are hydrolyzed first. Crystalline zones are protected by a "cage effect". When the amorphous domains depolymerize, cracks develop and paths open for penetration by hydrolyzing agents, which can attack crystalline domains causing an accelerated release of monomers (Chu, 1985). In sutures, the transition from amorphous to crystalline phase hydrolysis takes place at about W3 in vitro (Chu, 1985). It is likely that the process is slower for the dense (low S:V) bone screws observed by Böstman (1991) than for the suture threads described by Chu (1985). However, one cannot assume a predictable transition pattern, since amorphous-to-crystalline transitions may occur spontaneously (Anderson, 1993).

Investigations of responses of bone to polymer implants have been conducted primarily through histomorphometry and histology. The methods employed parallel those used in evaluating so-called "incorporation" of porous ceramic implants (Holmes et al., 1986). Accordingly, Hollinger (1983) implanted a 2 mm-diameter, 1.25 mm thick disk of 80 kDa 50:50 PLG into rat tibia and found osteoblasts in the space between the recipient bed boundary and a dissolving implant by 7 days. Trabeculae had formed by 21 days when the implant was reduced to small "islands". They reported a lack of inflammatory response. Observation of capillaries was restricted, however, to beyond the first 28 days post-implantation.

The role of the vascular system in incorporation of polymer implants

Implantation adds a complication to normal wound healing of any tissue which is reflected in vascular responses. It is expected that the reactions of bone microvasculature to non-antigenic yet histoincompatible (as opposed to "minimally stimulative") polymers, would fall between its reaction to autogeneic and allogeneic implants. Formulating predictions of expected vascular behavior for bone is, however, problematic. The lack of quantitative studies on this subject for bone healing was noted above, and Glowacki (in Kusiak et al., 1985; in Hardesty and Marsh, 1990) has pointed out a general lack of information about vascular reactions during implant incorporation.

In previous investigations of PAHA erosion in bone, there apparently have been no reports quantitating vascular events during the incorporation process. It is generally accepted that PAHA dissolution in bone is more rapid in a highly vascular host bed (Hollinger and Battistone, 1986), but this prediction has not been tested. Reference has been made by a number of investigators to the presence of vessels and there has been considerable interest in inflammatory responses. Accordingly, Hollinger and Battistone (1986) found a transient inflammatory reaction to PLG at 72 hours in a rat or mouse muscle pouch, and Böstman et al. (1990) report the appearance of edema in orthopaedic patients at the implant site about 12 weeks post-implantation. Bouet et al. (1991) using laser doppler velocimetry in rabbit muscle, have tried to correlate blood flow and biocompatibility of a number of non-aliphatic polymers to develop a standardized in vivo biocompatibility test. They concluded that their technique had promise, particularly for detecting incompatibilities based on "interface" toxicity, as opposed to leached-product toxicity.

Inducing a pattern of incorporation from these observations is not trivial. One can, however, build reasonable hypotheses by inducing how implant decomposition will interact with normal wound healing from what is known about each process. In response to trauma, a cascade of healing events is initiated which brings macrophages to the wound focus where they elaborate fibrogenins and angiogenins. Lactic acid, which is normally present in the anaerobic environment, stimulates macrophages to secrete angiogenic factors, but pyruvate and low pH per se do not (Jensen et al., 1986). Low pH increases blood supply (Davis and Wood, 1993), bringing in more O2 to allow oxidation of metabolites. Oxidation, and the release of buffering macromolecules from the numerous large pores, at this stage of healing (Renkin, 1988), will raise the pH. As pH rises, macrophages depart, lowering free angiogenin concentration and, consequently, the number of vessels. A decrease in O2 and blood supply ensues. Osteoblasts/osteoprogenitors attracted to the wound site by the release of "stormones" from damaged matrix, secrete collagen I.
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(Brighton et al., 1992). Where \( O_2 \) concentration and pH fall below a critical level, osteogenesis commences (Brighton et al., 1991), presumably accompanied by angiogenesis and mineralization. Angiogenesis at this juncture may be stimulated by the release of stormone angiogenins, such as basic FGF from subendothelial extracellular matrix (Folkman, 1987) during vessel resorption. Both the low \( O_2 \) concentration and low pH at the start of trabecular regeneration stimulate microcirculatory autoregulatory mechanisms to increase blood flow in bone (Davis and Wood, 1993). Vascularity and bone apposition rate are positively correlated in the BCI model as they are in other models (Heppenstall, 1980).

Vessel permeability selects which buffers or polymer-degrading agents diffuse to the implant environment, and together with the lymphatics, selects the metabolites and monomer products from erosion which are removed.

Observations of LP/V (Winet and Bao, 1991), suggest large pore incidence was greatest in healing bone trabeculae during maximum apposition, which occurred between W4 and W6 (after deposition of the collagen I matrix). During this period of maximum osteogenesis, angiogenesis brought in more new (small caliber) vessels (Winet et al., 1990a,b) which, in bone, exhibited more albumin-sized buffer extravasating pores (Winet and Bao, 1991). A rise in local pH resulted (Brueton et al., 1993), as mature remodeling bone was becoming established.

Small pores, which occupy at least three orders of magnitude more of the vascular surface appeared, from preliminary data, to be less permeable at W4. However, the fact that the vessel yielding this data was in fibrovascular tissue must be kept in mind for any interpretation of these data.

The reported effects of lactic acid on macrophages suggest a greater angiogenic response to PLA or high-PLA fraction PLG than to PGA, but no such comparison has been reported. The high incidence of large pores during inflammation (Renkin, 1988) suggest an increase in pH following an initial drop due to hypoxia and cell death (Brueton et al., 1993). As pH increases, there should be an acceleration of polymer erosion, an effect similar to that at low pH, but not autocatalytic, as has been reported for PLG (Chu, 1985). As long as buffering is maintained there will be a diffusion gradient for transport of monomers from the polymer. To the extent that macromolecular buffering agents from the microvascular accelerate polymer erosion, the existence of two large pore incidence peaks suggests two periods of attack on the implant, independent of any effect polymer erosion may have on the host.

Missing from the above account are macrophages, giant cells and polymorphonuclear leucocytes (PMNLs) which attack implants, and local edema from increased microvascular permeability which dilutes the interstitium, enhancing the exchange pool for degradation products.

**Measures of defect healing through eroding PLG, incorporation in the BCI BCI**

Traditional studies of PLG incorporation have been histological. Few have applied quantitative methods to study bone defects filled with PLG plugs and harvested at various time intervals to assess the changes in cell and tissue populations (Hollinger, 1983). Bone apposition was quantitatively and vascularily qualitatively evaluated in these studies. Results indicated a PLG-generated enhancement of bone healing (Hollinger, 1983).

The BCI appeared to be an ideal tool for studying incorporation of erodible polymers in bone. The BCI houses a slit-gap, essentially a culture cell with culture medium supplied by the organism. Its location in bone guaranteed that the same site was being sampled each week. Direct comparisons could be made with chronic *in vitro* studies of degradation of the same polymer. Before a valid interpretation of observations is possible, however, limitations of the BCI model for revealing implant-tissue interactions must be understood.

Materials placed in the slit-gap are not, initially, in contact with bone. They are merely imbedded in the medullary canal cells and fibrin-platelet matrix of a hematom. This is not to say that bone growing into the slit-gap is significantly stress-shielded. While the titanium screw obviously distorts the distribution of stresses exhibited during normal gap-healing, it does not affect osteogenesis sufficiently to prevent ingrown bone from persisting for up to two years (Winet and Hollinger 1993a; Winet et al., 1995). During incorporation, however, mechanical interactions of any material in the slit-gap with surrounding bone are delayed until apposing trabeculae contact the device. Thus, the BCI model essentially isolates chemical from mechano-chemical interactions between implant material and host tissue, at least in the early stages of healing. BCI data, consequently, give insight to the purely chemical interaction of eroding implant with host tissue during incorporation.

To date, only PLG has been subjected to BCI IVM. Samples were obtained from an extruded 100 \( \mu m \)-thick monofilament thread of \( M_w = 84 \) kDa, 50:50 (PLA: PGA) molar ratio material prepared by the Ethicon division of Johnson & Johnson. The lactide component of the copolymer was poly-l-lactide (PLLA). The thread was not reprocessed to control non-uniformities. Thus, microscopic examination revealed regions of slubbing (i.e., bulges).

It was hypothesized for this study that bone apposition rate (\( \Delta % B/t \)) would not be altered by PLG erosion.

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It was reasoned that in the Hollinger (1983) studies, healing rat bone tissue was able to use the PLG plug as a scaffolding. Moreover, the intimate contact between bone and plug shortened the distance invading vessels had to traverse with their acid-buffering plasma. In the BCI, there was a space of about 1 mm between the perimeter of the bur-hole and the PLG, the total defect diameter was 4 mm, that would probably serve as a reservoir for the accumulation of lactic and glycolic acid until blood vessels could cross into the slit-gap field-of-view. The PLG used was a 100 μm diameter thread, a poor scaffolding candidate in a 2 mm-wide tissue space, even when penetrated by advancing vessels. The interaction of PLG with tissue was postulated, consequently, to be more chemical than mechanical. Accordingly, it was predicted that the acidic monomers of PLG degradation could accumulate in the reservoir sufficiently (well beyond angiogenesis-stimulation levels) to inhibit neo-osteogenesis from the cortex. At this point, the report by Vasenius et al. (1992) showing that, except for a point well after healing was completed, blood pH changed insignificantly around polylactide intramedullary rods, had not appeared.

The two conflicting predictions suggested a balance between agents that favor and those that inhibit neo-osteogenesis. Consequently, it was hypothesized that their effects would offset each other, and neo-osteogenesis into a BCI loaded with PLG would occur at the same rate as in controls (i.e., unloaded BCIs); which is, in effect, the null hypothesis.

For the in vitro standard study, segments of the thread were placed in Tyrode’s solution at 37°C and photomicrographed daily the first week, and weekly from then on. The first apparent changes occurred within 8 days. They were: (1) an apparent thickening of the thread which may have been accompanied by shortening, and (2) increase in optical density. It took six weeks for significant fracturing to appear and at least 18 weeks for the remnants to be reduced to shards.

For the in vivo studies, thread segments, about 4 mm long, were cut off and placed between the two quartz windows of each chamber as it was assembled. The first image in Figure 4 shows the loaded segment. Care was taken to compress each segment only enough to keep it from slipping out of the slit-gap. The loaded BCIs were sterilized with ethylene oxide gas at room temperature for 12 hours and degassed for 24 hours using laminar-flow filter-sterilized air. Implantation, exposure and animal maintenance was the same as for the controls described above.

Observations commenced at W3 and two experimental groups were immediately identified. In the first group, the PLG appeared to have unravelled. Some vessels were present, but characteristically limited to the periphery of the field-of-view. This result was attributed to the existence of highly amorphous sections of the co-polymer and serves as an indicator of variability in thread structure. No bone trabeculae appeared and vascularization was markedly reduced in these BCIs as compared with controls over the course of the study.

In the second group, erosion, neo-osteogenesis and neo-angiogenesis appeared to progress in an ordered fashion. Both results have been reported (Winet and Hollinger, 1993a,b). A representative pattern of this group is shown in Figure 4. Trabeculae usually appeared at W5, occasionally being delayed to W6 or W7. Osseous filling of the slit-gap was rarely achieved before W10 and the maximum neo-osteogenic rate occurred between W7 and W8. The delay in comparison with the controls was statistically significant between W5 and W9. After this delay, however, osteogenesis, as indicated by the curve slope, proceeded at the same rate as the controls. An apparent "recovery" of osteogenesis after W5 produced apposition rates as high as 4.3%, a value 20% higher than the fastest control rate. Such accelerations were not, however, sufficiently characteristic to generate a steeper regression curve.

There was no evidence of incompatibility between trabeculae and polymer. The healing path of regenerating bone did not appear to be significantly altered by the polymer as indicated in Figure 5. Confirmation of this observation at the cellular level awaits further histological studies at higher magnifications.

Neo-angiogenesis analysis is still in preliminary stages but it was apparent that L/V versus time was delayed in the presence of PLG. Maximum perfused vascularity occurred between W10 and W11, three weeks after the control peak. Neo-angiogenesis was depressed, with the minimum L/V value almost halved. Nevertheless, the pattern of neo-angiogenesis persisted, including a projected maximum L/V prior to W3. Thus, the agreement of these results with control observations of Brighton et al. (1991), that osteogenesis is enhanced by low PO₂ and collagen synthesis by high PO₂, indicates that PLG incorporation does not alter basic physiological relationships. At this time, separate evaluation of trabecular and fibrovascular L/V has not been performed. It appears, however, that while the delay in neo-angiogenesis can be explained as an effect of trabecular apposition delay, the cause of its inhibition is more elusive. Further analysis will be necessary to determine if vascularity in the trabeculae has significantly changed in the presence of eroding PLG.

No correlation could be found between the disappearance of PLG and rate of neo-osteogenesis or neo-angiogenesis. The advantage of a chronic model like the BCI, is that each rabbit serves as its own reference. Resulting correlations were, accordingly, animal-specific.
Figure 4. Haversian healing (bone is from cortex) into a polymer-bearing bone chamber slit-gap compartment. First panel shows polymer pre-implantation. Other panels are vertical pairs of brightfield and fluorescent images. Times (W3, etc.) are in weeks post-implantation. Polymer (P), trabeculae (B) and fibrovascular tissue (F) are identified in the first bright-field row and can be discriminated in panels that follow. Perfused vessels are parallel with the long bone axis. W10 is represented with two fluorescent images, with the upper showing oxytetracycline deposition at mineralization sites (arrow). From Winet and Hollinger (1993) with permission.

and, consequently, a more accurate reflection of cause-and-effect.

Biocompatibility appeared to be supported by neoangiogenesis patterns. There was often evidence of vessel penetration of polymer fractures (Figure 4). Nevertheless, neo-osteogenic and neo-angiogenic delay during PLG erosion was significant. There is an apparent paradox between this circumstantial relationship and the lack of correlation between the disappearance of PLG and rates of the two regeneration processes cited above. The paradox may be resolved by postulating that one of the two apparently time-dependent processes is not truly
time-dependent. Since tissue regeneration is demonstrably time-dependent (Winet et al., 1993a,b), the candidate must be the PLG. A scenario in which the visible erosion of polymer is not representative of the actual loss of mass is not difficult to fashion for bulk eroders such as PLG. Visible erosion readings are based on changes in the polymer’s profile. Bulk eroders degrade internally before their outer boundary, which apparently acts like a semi-permeable membrane (Vert et al., 1992). Accordingly, monomer effluxes through the "membrane", which results in polymer weight loss and may influence tissue regeneration, but is not visible as surface changes on the device. This shortcoming of the BCI model may be surmounted by incorporating a covalently bound fluorescent dye in the polymer that would be released during depolymerization.

The retardation of bone and vessel regeneration did not appear to be caused by geometry of the PLG in the slit-gap field, because there was sufficient room for both vessels and bone before either contacted the polymer surface. Moreover, a persistent effect would have diminished, rather than delayed, the two regenerations. The fact that neo-osteogenic rates "recovered" following their delay, and neo-angiogenic plots retained their two-peaked pattern, supports the conclusion that normal homeostatic wound healing mechanisms were intact in the slit-gap tissue. Although the PLG thread segments were too small to exert pressure on the surrounding tissue when expanding, they could have deposited particles during erosion which are analogous to "wear" particles from prostheses. Accordingly, there remain some mechanical effects which cannot be ruled out.

Of the possible chemical effects, acidosis is not supported by recent observations on PLA in vivo (Vasenius et al., 1992) and PLG in vitro (Li et al., 1990a,b,c). However, pH in the PLA study was measured in nearby blood vessels rather than near the eroding surface. Thus, there is as yet no accounting for relatively unbuffered, local effects. In the present study, the expected stimulation by lactic acid monomers of neoangiogenesis did not materialize. A reduction of macrophages by W3, which typically occurs following the clearance phase (removal of necrotic tissue and clot) of healing, may have been the reason. In any case, bio-incompatibility, much less toxicity, did not appear to be a significant factor in these studies. This concurs with other rabbit studies (Vasenius et al., 1992). Specific chemical interactions yet to be investigated include the role of blood-borne agents and their rate of delivery on the erosion process. Accordingly, analysis of permeability and blood supply in the BCI-PLG model are currently in progress to obtain insight into the transport
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aspects of the erosion of this copolymer.

Given that degradation mechanisms for erodible implants are dominated by macrophage/giant cell and bathing fluid, the necessity to understand the role of microcirculatory responses to and effects on the synthetic guest are clear. Biocompatibility can be assessed in terms of speed and degree of perfused vascularization of the polymer, and resolution of the polymer (as a foreign body) can be described in terms of the state of the microcirculation at the time of implant disappearance. In order to achieve these assessments, one must either sacrifice a large number of subjects over short intervals to obtain large sample sizes at many time points or chronically sample a relatively small number of subjects.

BCI-IVM provides the latter approach with its inherent advantage of normalization. In addition to providing a defined group of subjects, it provides a defined tissue. The same 2 mm compartment is being observed each time. In essence, one has simultaneously a closed system in terms of location and an open system in terms of exposure to the integrated organism; a culture cell with natural medium, as it were. The slit-gap provides a cell into which any implant material may be placed for evaluation. On the down side, a bone screw does not allow truly "normal" healing and a gap/defect is not a fracture. The distortions, however, are almost exclusively mechanical.

Accordingly, chemical and carefully delineated mechanical conclusions may be drawn about the physiology of implant incorporation from BCI IVM analysis. In the case of PLG, it is evident from the evidence presented that the recipient host bed recognizes the implant as foreign. It does not, however, undertake a defensive (= inflammatory) response. There is, instead, a "cautious" healing. What remains to be determined is:

(1) the specific vascular permeability changes associated with the state of the polymer and,

(2) the degree to which the observed responses can be generalized to other preparations of PLG; and

(3) the details of tissue responses at the cellular level.

Future directions for application of BCIs to the study of implant-host interaction are suggested by the nature of the model. Any material that can be formed to fit the BCI slit-gap, including non-erodibles, such as titanium, can be tested for biocompatibility. The data produced from these observations can provide clinically relevant and basic knowledge about physiological responses to implant materials.

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During this study, the investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication #86-23, Revised 1985).

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Discussion with Reviewers

H. Plenk: With respect to soft and hard tissues repairing such a cortical bone defect, the "fibrovascular" granulation tissue invading the defect already contains vessels which never grow "within" but are already "between" the now "ossifying tissue". The resulting woven bone trabeculae can only "apose" on old pre-existing bone structures (e.g., the cortical bone border of the defect), but otherwise spread out in the defect between the vessels. Only then, lamellar bone is "apposed" on the trabeculae, forming eventually "primary osteons" or "plexiform bone". However, that will reduce the vascularized spaces, making it thus, unlikely that "apposing trabeculae are more vascularized than the (fibrovascular) tissue they replace". On the other hand, if new vascular channels in secondary osteons develop in such a situation, this is due to bone (and vascular) remodeling.

Author: The initial cortical bone bordering the defect is necrotic from burring friction heat. The initial tissue in the defect is a blood clot of medullary origin. By W4, granulation tissue in the slit-gap has reduced its L/V value to a minimum. The L/V value in opposing trabeculae, mixed woven and lamellar bone according to polarized light images, is increasingly greater than the remaining granulation tissue from W4 until bone fills the slit-gap. Focusing shows that bone vessels are not only in the bone but, they follow "Haversian" canals (because alignment is distinctly parallel with the long bone axis). These observations transcend interpretation. The origin of trabecular and vasculature may certainly be speculated upon and the suggestion that L/V increase occurs after trabeculae have advanced agrees with observations only if remodelling is as fast as apposition.

Y. Ohta: What do you consider to be the differences between "vascularity" and "vascularization"? So far few investigators have elucidated them. I think "vascularization" is suitable for this paper.

Author: "Vascularity" is the amount of vasculature present at any point in time. It is a noun. "Vascularization" is a transitive verb describing the process of occupying a volume of tissue with vessels. During wound healing, surviving vessels plus vessels vascularizing replacement tissue add up to the vascularity of the entire tissue volume. Vascularization usually occurs by angiogenesis. However, one can also vascularize a recipient bed with a vascular graft.

H. Plenk: How can a biodegradable polymer be "incorporated", especially in bone? In my experience, I never observed active new bone formation onto a polymer surface, so how you can make such a statement without presenting histomorphological evidence?

Author: The criticism is well taken. However, the term "incorporated" is used in the present work in the same sense that one "incorporates" food after digestion, i.e., at the molecular level. Since both α-hydroxy acids from the degrading copolymer can eventually enter the TCA cycle, they become part of the intermediary metabolism. In the same vein, one could challenge the use of the term "osteoconduction" which has appeared in the literature applied to bioerodible polymers.