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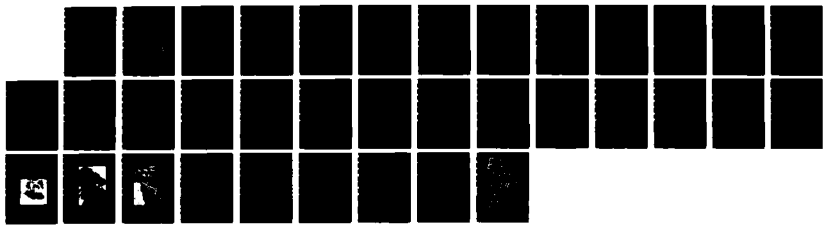

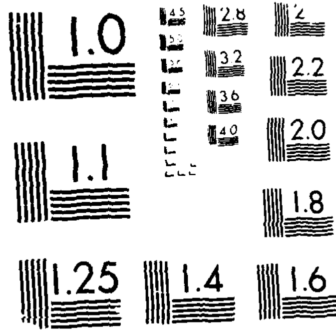

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FINAL REPORT

DEVELOPMENT OF A CALCIFIABLE MATRIX FOR BONE FORMATION

Dan W. Urry, Ph.D.

September 1, 1987

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Supported by

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As a contractual, development-synthetic effort, this project had two goals. The first was the delivery to the USAIDR of approximately 13 grams of synthetic peptide for *in vivo* study of calcifiability, ossification, biocompatibility and wound repair issues. The second goal was the characterization of the peptide in matrix form with respect to its *in vitro* calcification, stress-strain properties, biocompatibility and chemotaxis potential.

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FOREWORD

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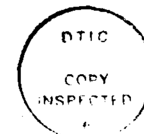

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TABLE OF CONTENTS

Materials Sent to the USAIDR .... 3 - 4

Synthesis and Cross-Linking 4

Matrix Characterization .... 5 - 10

Introduction .... 10 - 11

Materials and Methods .... 11 - 12

Results .... 13 - 14

Discussion .... 14 - 17

Matrix Characterization: Chemotaxis 17

Redirection: Biomaterials Development for Wound Repair .... 17 - 18

Table 19

Chart 20

Legends for Figures 21

Figures .... 22 - 28

References .... 29 - 31

Distribution List 32

This project was developmental in nature. Its objective was the elaboration of an "off the shelf" matrix for bone formation with the approach being based on the polypentapeptide, HCO-(Val_1-Pro_2-Gly_3-Val_4-Gly_5)$_n$-OME (PPP). This polymer, when cross-linked into an insoluble matrix, calcified massively on exposure to normal human serum dialyzates. The goal of the Laboratory of Molecular Biophysics was twofold: one, the synthesis of such a material in quantity and its subsequent forwarding to the USAIDR for *in vivo* testing for calcification and ossification and two, the characterization of the material before and after *in vitro* calcification prior to the *in vivo* assays.

Materials Sent to the USAIDR

Approximately 13 grams of polypeptide were synthesized and forwarded to the USAIDR. In the first phase of the synthetic program, 0.8 grams of the polypentapeptide was sent in September, 1983. This included 400 mg of PPP cross-linked with 10 MRAD and 400 mg of PPP cross-linked with 20 MRAD. The remaining irradiation cross-linked material consisted of two dry strips - a lengthwise cut (10 MRAD) and a crosswise cut (15 MRAD). Delivered in September and December, 1984 was the second phase material. This consisted of 5 grams of the PPP in dry form, 2 grams of PPP in its coacervated form, 1.0 gram of the PPP in matrix form prepared with γ-irradiation, 0.9 gram of the PPP in a calcified irradiated cross-linked matrix form and 1 gram of (Val-Gly-Pro-Gly-Val-Gly)$_n$ in dry form. In addition, 5 milligrams of the osteocalcin pentapeptide sequence, Phe-Tyr-Gly-Pro-Val, were sent. The third phase materials were sent in April, 1986 and their total weight was 2.16 grams. This consisted of 712 mg of γ-irradiation cross-linked (20 MRAD) PPP, 644 mg of γ-irradiation cross-linked (20 MRAD) PPP covalently coupled with polyhexapeptide

in a 9 to 1 ratio, and doped with 10^{-8} M hexapeptide and 737 mg of γ-irradiation cross-linked (20 MRAD) PPP covalently coupled with polynonapeptide (PNP) in a 9 to 1 ratio and doped with 10^{-8} M nonapeptide. In summary:

1st Phase Material	PPP 10 MRAD	400 mg	
(1983)	PPP 15 MRAD	400 mg	
		800 mg	0.8 gm
2nd Phase Material	PPP dry	5,000 mg	
(1984)	PPP coac.	2,000 mg	
	PPP 20 MRAD	1,000 mg	
	PPP 20 MRAD + Ca	900 mg	
	$(VGPGVG)_n$	1,000 mg	
	Phe-Tyr-Gly-Pro-Val	5 mg	
		9,905 mg	9.9 gm
3rd Phase Material	PPP 20 MRAD	712 mg	
(1986)	PHP-PPP 20 MRAD	712 mg	
	PNP-PPP 20 MRAD	736 mg	
		2,160 mg	2.16 gm
Total Amount of Material			12.86 gm

Synthesis and Cross-linking

Important to the development of an irradiation cross-linked readily calcifiable matrix was the synthesis of very high molecular weight polypentapeptide. The synthetic pathway was optimized with respect to yield and molecular weight which allowed the subsequent determination of optimal cross-linking conditions. This also facilitated the preparation of 0.8 grams of material in phase one and ~10 grams of material in phase two.

Matrix Characterization: Calcification, Young's Modulus, Biocompatibility

γ-irradiated high molecular weight PPP calcified *in vitro* when exposed to a serum diffusate plus/minus exogenous calcium and phosphate. The mineralization was positively correlated with increasing molecular weight, a property which could be due to better matrix filament orientation and minimal matrix perturbations at higher molecular weight. The cross-linked PPP also calcified *in vivo* ("A Synthetic Polypentapeptide of Elastin for Initiating Calcification," Hollinger, Schmitz, Yaskovich, Long, Prasad and Urry, in press, Calcified Tissue International). The following describes the Laboratory's efforts to optimize the PPP synthetic scheme and its calcification after matrix formation by γ-irradiation cross-linking.

D. W. Urry, S. A. Wood, R. D. Harris and K. U. Prasad, "Polypentapeptide of Elastin as an Elastomeric Biomaterial," In *Polymers as Biomaterials* (S. W. Shalaby, T. Horbett, A. S. Hoffman and B. Ratner, eds.) Plenum Publishing Corporation, New York, New York, 17-32, 1985.

In Summary: The polypentapeptide of elastin, $(\text{L·-Val}^1\text{-L·Pro}^2\text{-Gly}^3\text{-L·Val}^4\text{-Gly}^5)_n$, is synthesized with $\bar{n} > 200$. The purity of the synthesis is verified by carbon-13 nuclear magnetic resonance spectra. Coacervate concentrations are γ-irradiation cross-linked at 2,6,10,14,18,26 and 34 MRADs to produce elastomeric bands. The dependence of the elastic modulus on cross-linking dose is demonstrated and thermoelasticity studies indicate a dominantly entropic elastomeric force. These results are discussed in connection with the property of the polypentapeptide to self-align into fibers, in connection with a proposed entropy source for the elasticity utilizing a class of β-spiral conformations and in terms of specific analogs which had been synthesized to test concepts of conformation and entropy source. Finally, the biocompatibility of the polypentapeptide is considered.

D. W. Urry and K. U. Prasad, "Syntheses, Characterizations and Medical Uses of the Polypentapeptide of Elastin and Its Analogs," In Biocompatibility of Tissue Analogues (D. F. Williams, ed.) CRC Press, Inc., Boca Raton, Florida, 89-116, 1985.

In Summary: It has been found by Sandberg and co-workers that the precursor protein of fibrous elastin contains repeating peptide sequences. The dominant repeat sequences are a polypentapeptide (PPP) $(L{\cdot}Val^1\text{-}L{\cdot}Pro^2\text{-}Gly^3\text{-}L{\cdot}Val^4\text{-}Gly^5)_n$, a polyhexapeptide (PHP) $(L{\cdot}Ala^1\text{-}L{\cdot}Pro^2\text{-}Gly^3\text{-}L{\cdot}Val^4\text{-}Gly^5\text{-}L{\cdot}Val^6)_n$, and a polytetrapeptide (PTP) $(L{\cdot}Val^1\text{-}L{\cdot}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$. The value of n for the PPP is 11 in pig and 13 in chick (the latter being without a single variation); the value of n for the PHP is greater than 5 for pig; the value for n for the PTP is 4. The monomers, oligomers and high polymers of these repeats have been synthesized and conformationally characterized. The high polymers have been cross-linked and the cross-linked PPP and the cross-linked PTP have been found to be elastomeric and capable of an elastic modulus similar to that of the natural elastic fiber. The PHP forms cellophane-like sheets and is proposed to assume a precross-linked aligning and interlocking role between protein chains in the fiber. Conformational studies using, in addition, cyclic analogs confirmed in the PPP the presence of a recurring secondary structural feature called a "β-turn" and have led to a new concept of elasticity referred to as a "librational entropy mechanism" of elasticity. PPP analogs in which the Gly residues have been replaced by L•Ala and D•Ala residues substantiate the new concept of elasticity and result in additional new biomaterials.

Accordingly, the PPP, the most striking primary structural feature of the precursor protein of the elastic fiber, represents a new type of elastomeric biomaterial which is of direct interest and which can be modified in

interesting ways. Two intriguing new perspectives have recently become evident. One is that the synthetic elastomeric polypeptide biomaterial can be so constructed as to become covalently cross-linked by tissue enzymes to newly synthesized connective-tissue protein. This is because lysyl oxidase, the natural extracellular cross-linking enzyme, will cross-link PPP chains in which occasional lysine residues have been introduced in position 4. The second new perspective is that the synthetic polypeptide biomaterial can directly be a source of chemotactic peptide for inducing cellular migration into the biomaterial. This derives from the demonstration that the permutation of the hexamer, L•Val-Gly-L•Val-L•Ala-L•Pro-Gly, is a potent chemotactic peptide for elastin-synthesizing fibroblasts. Thus, a synthetic polymer containing the PHP and the PPP in series in a single chain, once cross-linked, and containing occasional lysine residues, would have interesting structural and cellular effector properties for a biomaterial. The biomaterial could induce migration to its matrix of elastin-synthesizing fibroblasts, and it could become chemically cross-linked to the tropoelastin elaborated from the fibroblasts so induced into the material.

Before such perspectives can be realized, however, there are a number of technical problems that require solving: there is the formation of the cross-linked matrix; there are the required characterizations along the way; there are questions of biocompatibility to be addressed; there are perspectives of varied and directed uses of the biomaterial that can be considered. In the present review, the primary problem of synthesizing large quantities of high-molecular-weight PPP at reasonable cost is discussed in terms of a synopsis of the approaches that have been tried, and the present best approach is given. Both chemical and γ-irradiation cross-linking approaches for matrix preparation are presented. Several useful means of verifying the product and characterizing both the high polymer and the cross-linked matrixes are included.

Finally, there is reference to tests for biocompatibility and to means of adapting the basic PPP matrix to selected uses.

K. U. Prasad, M. A. Iqbal and D. W. Urry, "Utilization of 1-Hydroxybenzotriazole in Mixed Anhydride Coupling Reactions," Int. J. Pept. and Protein Res., 25, 408-413, 1985.

In summary: The coupling of Boc-Val-OH to either H-Pro-OBzl or H-Pro-Gly-Val-Gly-OBzl by the mixed anhydride method leads to the formation of a urethane by-product in yields of 40 - 60%. This side reaction can be suppressed by the addition of HOBt to the reaction mixture before the amino component is added. This results in a substantially increased yield of the desired peptide.

S. A. Wood, K. U. Prasad and D. W. Urry, "Cross-Linked Polypentapeptide of Elastin as a Calcifiable Matrix: Molecular Weight Dependence," Calcif. Tissue Int., 37, 565-571, 1985.

In Summary: The polypentapeptide, $(L{\cdot}Val^1\text{-}L{\cdot}Pro^2\text{-}Gly^3\text{-}L{\cdot}Val^4\text{-}Gly^5)_n$, when crosslinked by γ-irradiation was shown to calcify when exposed to dialyzates of calcium and phosphate augmented fetal bovine sera and the molecular weight dependence of this calcification is investigated. Five molecular weight fractions, labeled I to V in order of increasing polymer size from under 12,000 dalton (I), that is, $\bar{n} < 30$, to over 100,000 daltons (V), that is, $\bar{n} > 240$, were γ-irradiation cross-linked at 10 - 12 MRAD to form matrices I - V. Calcium-45 was used to follow the time course and relative amount of calcium uptake from the sera. Scanning electron microscopy and electron probe microanalysis were used to characterize the extent of matrix calcification. All matrices took up calcium-45 from the sera; however, only matrices formed from polypentapeptide with $\bar{n} > 100$ calcified, that is, matrices III, IV and V. Matrix V with $\bar{n} > 240$ calcified massively and in a manner comparable to chemically cross-linked polypentapeptide with $\bar{n} \sim 40$ using nonaugmented sera.

Presumably, γ-irradiation results in chain breakage. The γ-irradiation cross-linked matrices with values of $\bar{n}$ ranging from under 30 to greater than 240 establish the molecular weight dependence of matrix calcifcation.

S. A. Wood, J. E. Lemons, K. U. Prasad and D. W. Urry, "In Vitro Calcification and In Vivo Biocompatibility of the Cross-Linked Polypentapeptide of Elastin," J. Biomed. Mater. Res., 20, 315-335, 1986.

In summary: The in vitro calcifiability and molecular weight dependence of calcification of the polypentapeptide, $(L{\cdot}Val^1\text{-}L{\cdot}Pro^2\text{-}Gly^3\text{-}L{\cdot}Val^4\text{-}Gly^5)_n$, which had been γ-irradiation cross-linked have been determined when exposed to dialyzates of normal, nonaugmented fetal bovine serum. The material was found to calcify: calcifiability was found to be highly molecular weight dependent and to be most favored when the highest molecular weight polymers ($n \approx 240$) had been used for cross-linking. The in vivo biocompatibility, biodegradability and calcifiability of the γ-irradiation cross-linked polypentapeptide were examined in rabbits in both soft and hard tissue sites. The material was found to be biocompatible irrespective of its physical form and to be biodegradable but with n of 200 or less it was not shown to calcify or ossify in the rabbit tibial nonunion model.

J. O. Hollinger, J. P. Schmitz, R. Yaskovich, M. M. Long, K. U. Prasad and D. W. Urry, "Synthetic Polypeptide of Elastin for Initiating Calcification," (in press) Calcif. Tissue Int.

In summary: A polypentapeptide (PPP) of tropoelastin having a repeating amino acid sequence of $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$ was evaluated for its potential to initiate in vivo calcification and to enhance bone formation in non-healing calvarial wounds (8.0 mm) in 396 adult Walter Reed rats. There were four configurations of the PPP (molecular weight range of 50K-100K dalton) consisting of 1-dry PPP, 2-coacervate PPP, 3-gamma irradiated, cross-linked PPP, 4-calcified, gamma irradiated, cross-linked PPP. These four iterations plus a

control group of animals constituted the five treatment classes that were evaluated at days 1, 3, 7, 21, 42, and 147. There were 72 rats used for each treatment and 36 rats for the control. Following euthanatization, specimens were placed into 70% ethanol, embedded in polymethyl methacrylate, sectioned at 3.5 micrometers, and alternating sections were stained with Masson-Goldner trichrome and von Kossa stains.

Histomorphometric analysis was accomplished using a Zeiss Universal microscope (250X) and Videoplan Image Analysis System to evaluate five random histologic fields from margin to margin of the craniotomy. Trabecular bony volume and area of calcification islands were quantitated. A Student's t-test for unpaired data to determine between treatment differences (within the same temporal groups) revealed that there was no significant difference between treatments and control for trabecular bony volume; however, there was a significant difference between experimentals and control for calcification islands ($p < 0.05$) such that calcification islands for the experimentals were greater than the control. There was not a significant difference between experimental treatments. These data provide the first *in vivo* demonstration of the validity of the neutral site binding/charge neutralization mechanism of calcification. Although the synthetic peptide did support calcification, in no group was new bone formed in the craniotomies.

KEY WORDS: Polypentapeptide--Calcification--Histomorphometry--Bone Repair.

INTRODUCTION

Numerous investigations have been conducted using a variety of implant materials for osseous wound repair. Traditionally used agents such as autogenous and allogeneic banked bone have been reported to have a failure rate that

ranges from 13-30% (1). Alternatives, therefore, have been sought. Alloplastic agents such as the ceramics and biodegradable polymers, along with bone derivatives and bone matrix proteins have been evaluated for application in osseous repair (2-7). A calcification initiator, diphosphoinositide-lysozyme, was tested for a possible role in bone healing (5). In addition, a combination of the same initiator plus a biodegradable, biopolymer matrix of 50:50 polylactide-coglycolide was investigated for osseous regeneration (6). Histomorphometric evidence indicated a possible enhancement to the bone wound repair process. Other calcification initiators may find utility for regeneration of hard tissue. A polypentapeptide, $(\text{Val-Pro-Gly-Val-Gly})_n$, of tropoelastin (8,9,10) has been shown to calcify on incubation in dialyzates of normal fetal bovine serum after being flow oriented and weakly chemically cross-linked (11). This calcification is a surface and bulk property (11) as it is for its parent compound, elastin (11-14). The end product of this mineralization process is hydroxyapatite (15). It was the purpose of our study to determine if three different doses of four variations of the polypentapeptide could effect *in vivo* calcification of the synthetic polypentapeptide and could produce osseous healing in critical size craniotomies in rats.

MATERIALS AND METHODS

After achieving suitable anesthesia using sodium pentobarbital (3-5 mg/100 mg. of body weight, ip), craniotomy defects of 8 mm in diameter were prepared in the parietal bones of 324 adult Water Reed mixed sex rats using trephines in a dental rotary hand piece and copious saline irrigation. There were 4 treatment groups of 72 rats each. Twenty-four animals in Group 1 received either 3, 8, or 15 mgs of dry PPP. Group 2 similarly was treated with 3, 8, or 15 mgs of the PPP in coacervate form. Groups 3 and 4 were organized exactly the same as the

first two groups and treatments for 3 and 4 were irradiated, cross-linked PPP and irradiated, calcified PPP, respectively. The latter cross-linked matrix was precalcified in a serum based calcifying medium (12) for 40 days. Experimental material was placed on dura and within the skull defect margins (Figure 1a). Soft tissue was closed with surgical staples. The control group, consisting of 36 rats, received no implants in the craniotomies. Twelve rats (4 from each experimental dosage group) and six control animals, were sacrificed on days 1, 3, 7, 21, 42 and 147. At necropsy, the calvaria were exposed and each implant and control site was removed with 2-3 mm of surrounding host bone. Specimens were immediately placed into 70% ethanol, followed by ascending alcohols and then into polymethyl methacrylate for undecalcified sectioning to 3.5-4.0 micrometers. Consecutive serial sections were stained using a modified Masson-Goldner trichrome and von Kossa stains. Histological specimens were assessed for local host responses. Inflammation and cellular infiltrate associated with the immune system were noted by microscopic examination. Histomorphometric evaluation of new bone formation and calcification in the wound beds was accomplished using a Zeiss Universal microscope (250X) and a Zeiss Videoplan Image Analysis System. Trabecular bony volume (TBV) and calcification islands (CI) were quantitated by making five random tracing measurements across the largest diameter of the craniotomy using a digitizing board and light cursor. Data analyses for between group differences and differences over time were performed using two-way analysis of variance and a Student's t-test was used to determine between treatment differences (within the same temporal group).

The polypentapeptide was prepared with classical solution peptide syntheses methods as previously reported (16,17) and then a fraction was covalently cross-linked with gamma irradiation (16).

RESULTS

DAYS 1, 3, and 7.

Polymorphonuclear leukocytes were identifiable at day 1, diminishing in numbers from days 3 to 7. Some small round cells were apparent at days 3 to 7; however, there was no indication of an unusual or adverse tissue reaction to any iteration (at any dose) of the PPP. Infrequent plasma cells were identifiable at 7 days. Also at 7 days there was a flimsy connective tissue capsule of immature, loosely arranged connective tissue investing and isolating islands of PPP. Quantitation of calcification islands (CI) and trabecular bony volume (TBV) revealed no statistical difference in treatments versus control at 1 and 3 days; however, at day 7 there was a statistical difference between CI-control vs CI-of all PPP iterations. There was neither a difference between any of the PPP treatments nor was there a difference between TBV-control and TBV-PPP's (Figures 2 and 3). CI's were most abundant along the periphery of connective tissue enclosed islands of PPP. When data in Figure 2 are plotted, CI vs. time, (Figure 5) it is noted that calcification increases between days one and seven and then plateaus out. This rate of calcification compares favorably with previously published in vitro data for α-elastin, tropoelastin and the polypentapeptide (12, 28).

DAYS 21, 42, and 147

Neutrophylic leukocytes were few and small round cells, plasma cells and macrophages were rare. Multinuclear giant cells occasionally were seen located contiguous to the connective tissue capsule investing the PPP. Giant cells were observed to be both external to and within the capsule. There was no evidence at any time period of an adverse tissue reaction to any iteration or dose of the PPP. At no time period was there a significant difference in the amount of TBV that developed at the control and experiments sites and there was no indication

of an osseous heal across the 8.00 mm craniotomy wounds. There was a statistically significant difference in the CI-control versus all PPP interations and doses at 7, 21, 42, and 147 days. There was no difference in CI between any of the PPP treatments. The extent of calcification was greatest at the periphery of the connective tissue encapsulated islands of PPP and diminishing centripetally. There was no evidence of ossification within the PPP (Figs. 4a, b, and c). Minimal new trabecular or woven bone was apparent at the craniotomy margins.

DISCUSSION

In a prevous paper it was reported that a calcification initiator, diphosphoinositide-lysozyme, hastened bony repair in endochondral wound sites in rats (5). It was hypothesized that the diphosphoinositide-lysozyme possibly could function as a pseudo-matrix vesicle due to its component of phosphatidyl inositol. Wuthier maintains that calcification depends upon matrix vesicles that contain the acidic phospholipids phosphatidyl inositol and phosphatidyl serine (19). The PPP of elastin also initiates calcification *in vitro*, albeit via a mechanism distinctly different from that of matrix vesicles. It calcifies via the neutral site/charge neutralization mechanism (20). This was formulated in 1971 based on two observations: one - the elastic fiber in the arterial wall is the initial site for *in vivo* ectopic calcification (21) and two, elastin, the single protein of the elastic fiber, contains a high percentage of glycine (33%) and a higher still percentage of amino acid residues with hydrophobic groups (65%) (22). With few amino acids with functional side chains (23), it is not a likely candidate for calcium binding to charged sites. The neutral site binding/charge neutralization theory proposed that calcium ions bound first to formally neutral yet polarizable oxygens of the peptide bond carbonyls, this

would make the neutral elastin matrix positive which in turn would cause it to sequester polyvalent anions such as phosphate. Phosphate would neutralize the charge-charge repulsion between calcium ions so more calcium would bind, then more phosphate would bind until a cascading series of events led to calcium phosphate deposition and its conversion to hydroxyapatite. The validity of this mechanism has been established in vitro for elastin and the neutral polypentapeptide of elastin (11-15). With this background understanding, our rationale for using PPP at orthotopic bone wounds was based upon the polypentapeptide's ability to attract to the healing environment the necessary cations and anions associated with the ossification process. We anticipated that the accumulation of these requisite ions in the extracellular healing milieu might encourage bone repair in an otherwise non-healing, critical size defect (24).

The earliest efforts to make calcifiable matrices using the PPP of elastin utilized chemical cross-linking in which two different polymer syntheses were carried out. In one polymer synthesis, an occasional Glu residue was introduced in position four and in the second polymer synthesis, an occasional Lys residue was also included in place of the Val in position four. The two polymers were mixed and a water soluble carbodiimide was used to bring about cross-linking by amide formation utilizing the Glu and Lys side chains. The resulting matrix calcified more readily than the γ-irradiation cross linked matrices. While with this chemical cross-linking the yield of matrix was low, the result raises the possibility, for example, that an occasional Glu in position four might enhance the potential for the matrix to initiate calcification.

All variations and all three doses of the PPP were tissue tolerant. This tolerance is consistent with previous results demonstrating biocompatibility with human gingival fibroblasts, human skin fibroblasts, rabbit muscle and bone (25-28). The encapsulation of the PPP by connective tissue is similar to the

fibrous tissue capsule seen in soft tissue implants with rabbits (28). Also, all variations supported calcification at a significantly higher level than the control by the seventh day. These are the first data to establish the validity of the neutral site binding/charge neutral mechanism in vivo. However, osseous repair of the craniotomy sites was never fulfilled. By using histomorphometry we were able to quantitate the amount of CI and TBV. The graphs (Figures 2 and 3) were based upon an averaging of three different doses of the same iteration of PPP at the same time period. We did this because it was apparent that no significant difference existed between doses. Therefore, each value on our bar graph (plus standard deviation) represents an averaging of 60 fields: 5 random fields per rat, 4 rats per dose.

Although bone formation was not observed in these experiments, the concept of using a synthetically produced repeating elastin pentapeptide to elicit an osseous response could still be a viable approach if the appropriate state of the matrix were used. It has been shown that the extent of calcification of this matrix is dependent on the molecular weight of the polymer (29) and the irradiation doses used for cross-linking (30). The method of cross-linking may be important. Chemically cross-linked PPP matrices readily calcify massively and throughout their bulk (11) while gamma irradiation cross-linked material initially calcifies more slowly with concentration in discrete areas. Significant too, maybe the molecular orientation of the PPP. Rather than a cross-linked sample that is pulverized and dispersed, a carefully flow oriented matrix might be used. Also, bone formation may require a calcifiable matrix that is not as compacted or dense as was the material tested. The matrix could be made porous to facilitate cell entry.

It is possible, that a combination of PPP and osteoblast recruiting and/or differentiating factors may prove to be efficacious (16). For this purpose the

PPP could be combined with the pentapeptide Phe-Tyr-Gly-Pro-Val which is the carboxyl sequence of osteocalcin (18) and which was originally thought to be chemotactic for osteogenic cells (31). However, care must be exercised since experiments in our Laboratories have shown that osteosarcoma cells (32,33) are not chemotaxtic towards this synthetic peptide. Another peptide of note to test in conjunction with PPP would be the bone morphogenetic protein of Urist (7). Perhaps the activity of an effective osteoblast population will obviate calcification islands and rather, will be capable of packaging rapidly accumulating calcium and phosphate for use in the ossification process.

Matrix Characterization: Chemotaxis

Further matrix characterization evaluated the chemotactic potential of the C-terminal pentapeptide fragment of osteocalcin (Phe-Tyr-Gly-Pro-Val) towards osteoblast-like rat osteosarcoma cells and the chemotactic potential of the polyhexapentapeptide and polynonapentapeptide doped with the repeat hexapeptide and repeat nonapeptide of elastin respectively towards fibroblasts. In corroboration of results obtained by M. Somerman, but in contrast to results collected by G. R. Mundy, this Laboratory demonstrated that the pentapeptide, Phe-Tyr-Gly-Pro-Val, was not chemotactic towards osteosarcoma cells. These data showed that it was unwise to pursue the idea that this particular peptide could be added to the PPP to enhance invasion of bone forming cells via in vivo chemotaxis. The Laboratory did demonstrate that the polyhexapentapeptide and polynonapentapeptide were chemotactic for fibroblasts capable of producing elastin.

Redirection: Biomaterials Development for Wound Repair

Based on the results described above, it was decided during the September, 1985, USAIDR site visit to change the direction of the research effort

toward wound repair. The rationale for this new area of endeavor was based in the observation that normal scar tissue is rigid from over production and incorporation of collagen into the wound with a concomitant minimal contribution from elastin. Since the hexapeptide and nonapeptides of elastin are chemotactic for elastin synthesizing fibroblasts, their ability to induce elaboration of elastic fiber during repair would be reasonable to monitor. For this purpose, the Laboratory delivered to the USAIDR the third phase materials (2.16 grams of polypeptides, cf. page 1). Their in vivo properties are currently being tested by the USAIDR.

TABLE

TREATMENTS

1. poly H(Gly-Val-Gly-Val-Pro)n-OH

   > 50K dalton < 100K dalton

   DRY

2. poly H(Gly-Val-Gly-Val-Pro)n-OH

   > 50K dalton < 100K dalton

   COACERVATE

3. poly H(Gly-Val-Gly-Val-Pro)n-OH

   COMBINED: 15 Mrad + 10 Mrad
   Irradiation cross-linked

   >100K dalton

4. poly H(Gly-Val-Gly-Val-Pro)n-OH

   Irradiation cross-linked

   Precalcified

   < 100K dalton

5. Control

   Each treatment was broken down into a dose response trio consisting of 3, 8 and 15 mgs. There were 12 rats per time period evenly divided between the 3 doses. The exception was the control group where there was 6 rats per time period. The time periods consisted of 1, 3, 7, 21, 42 and 147 days. A total of 324 rats were used (288 experimentals and 36 controls).

CHART

TREATMENT* VERSUS TIME

TIME days	1	3	7	21	42	147	TOTAL
TREATMENT							
1	doses 3:4 rats (mgs) 8:4 rats 15:4 rats	"	"	"	"	"	72 rats
2	12 rats	"	"	"	"	"	72 rats
3	12 rats	"	"	"	"	"	72 rats
4	12 rats	"	"	"	"	"	72 rats
5	6 rats	"	"	"	"	"	36 rats
TOTAL:	66 rats				GRAND TOTAL:		324 rats

*Each time period consisted of 6 treatment groups with each group divided into a trio of doses, except for the control.

Legends for Figures

1. Fig. 1a: Schematic for inserting the polypentamer (PPP) into a standard sized non-healing calvarial defect and the six necropsy periods.

   Fig. 1b: Schematic for histomorphometry processing and analysis of undecalcified bone specimens.

2. Fig. 2: Graph 1: The percent total area of calcification islands (CI, %) (x +/- SD).

3. Fig. 3: Graph 2: The trabecular bony volume (TBV, mm^3/mm^3) (x +/- SD).

4. Fig. 4a: 42 days, tissue capsule (E) investing pentamer (*), host bone (B), fibrous tissue (F). (Goldner trichrome, 16 x)

   Fig. 4b: 42 days, same field as 3a showing numerous calcification islands within pentamer. (von Kosa, 16 x)

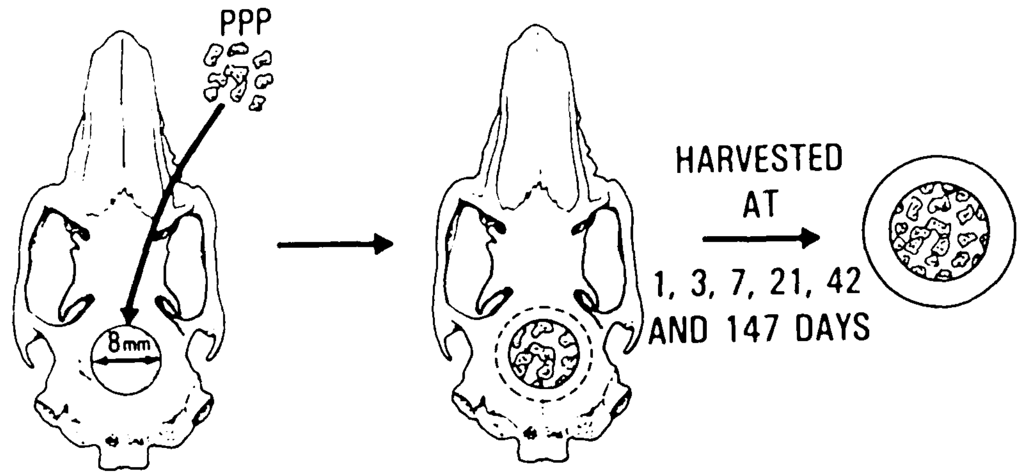
   Fig. 4c: 42 days, a higher magnification demonstrating host bone (B), calcification islands (C). (63 x)

5. Fig. 5: Calcification index as a function of time, replotted from data in Figure 3.

RAT CALVARIA

NECROPSY

PPP WITH 3 mm PERIPHERAL HOST BONE



PROCESSING OF PPP FOR HISTOMORPHOMETRY

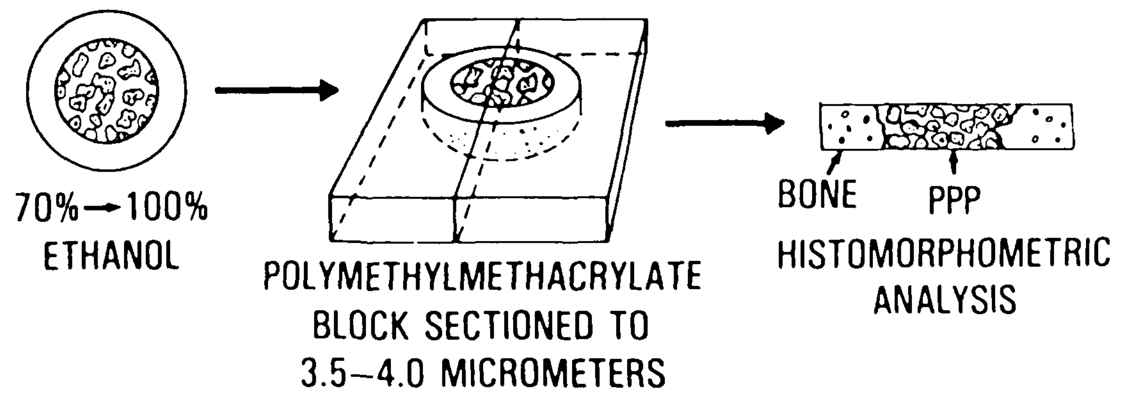
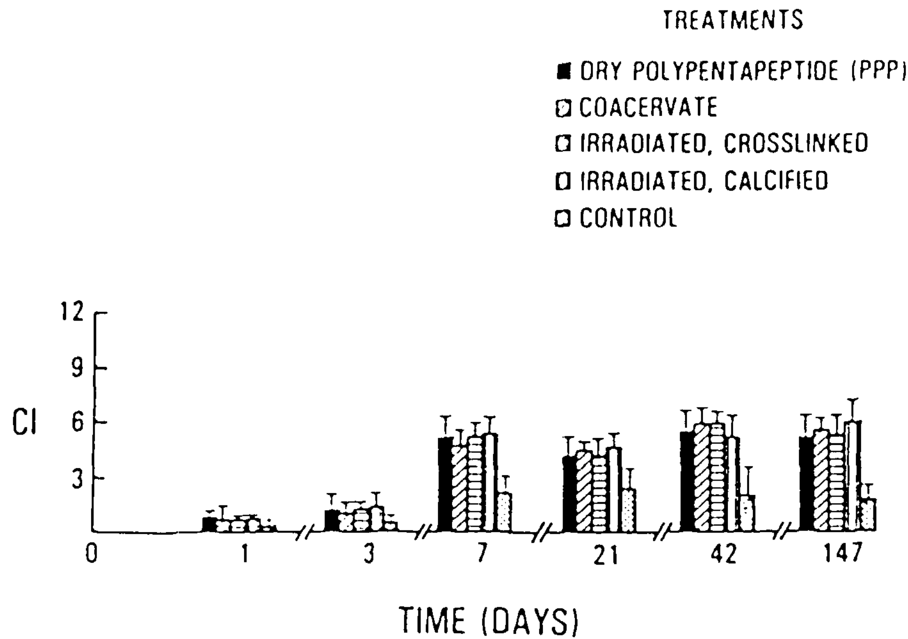


FIGURE 1

GRAPH 1

PERCENT TOTAL AREA OF CALCIFICATION ISLANDS (CI) (%)



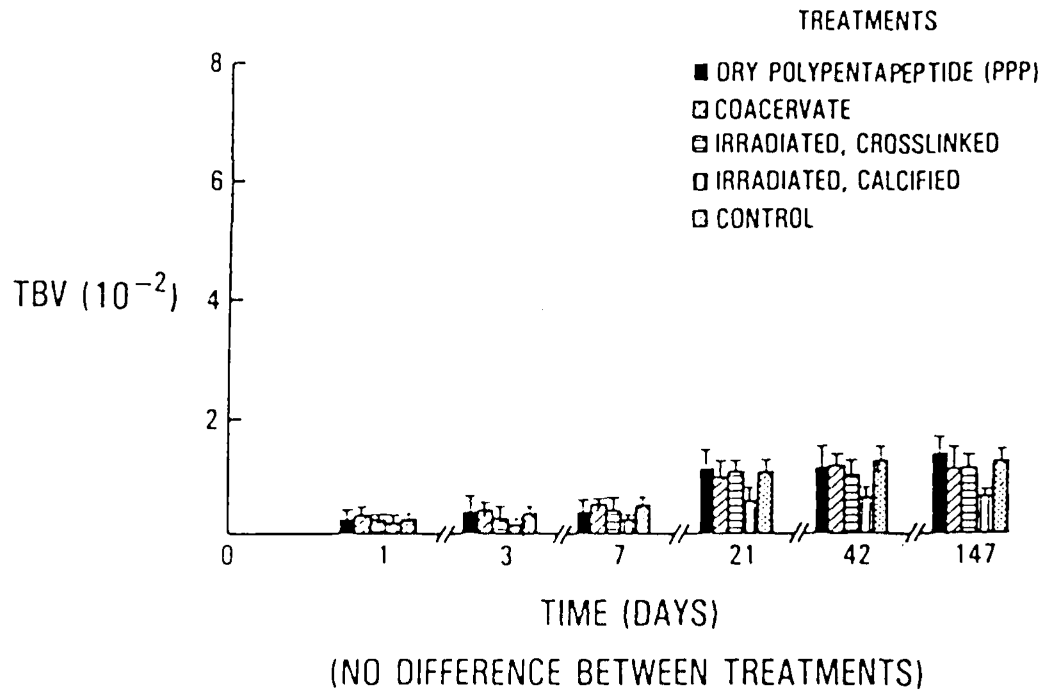
EXPERIMENTAL TREATMENTS AND CONTROL)
(SIGNIFICANT DIFFERENCE BETWEEN
EXPERIMENTAL TREATMENTS AND CONTROL)

FIGURE 2

GRAPH 2

TRABECULAR BONY VOLUME (TBV)

(mm^3/mm^3)



(NO DIFFERENCE BETWEEN TREATMENTS)

FIGURE 3

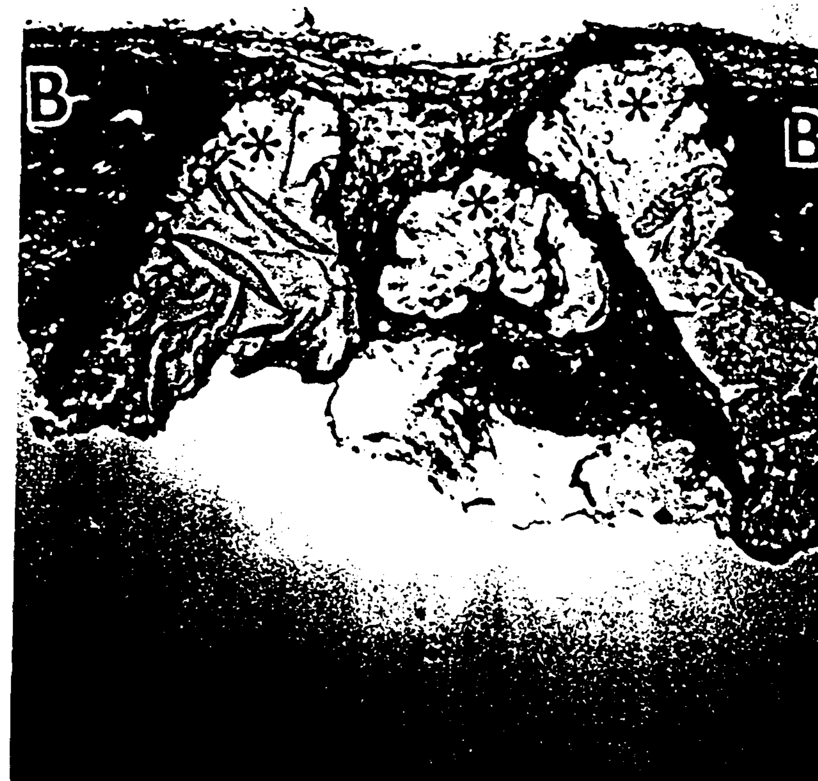


FIGURE 4A

FIGURE 4B

FIGURE 4C

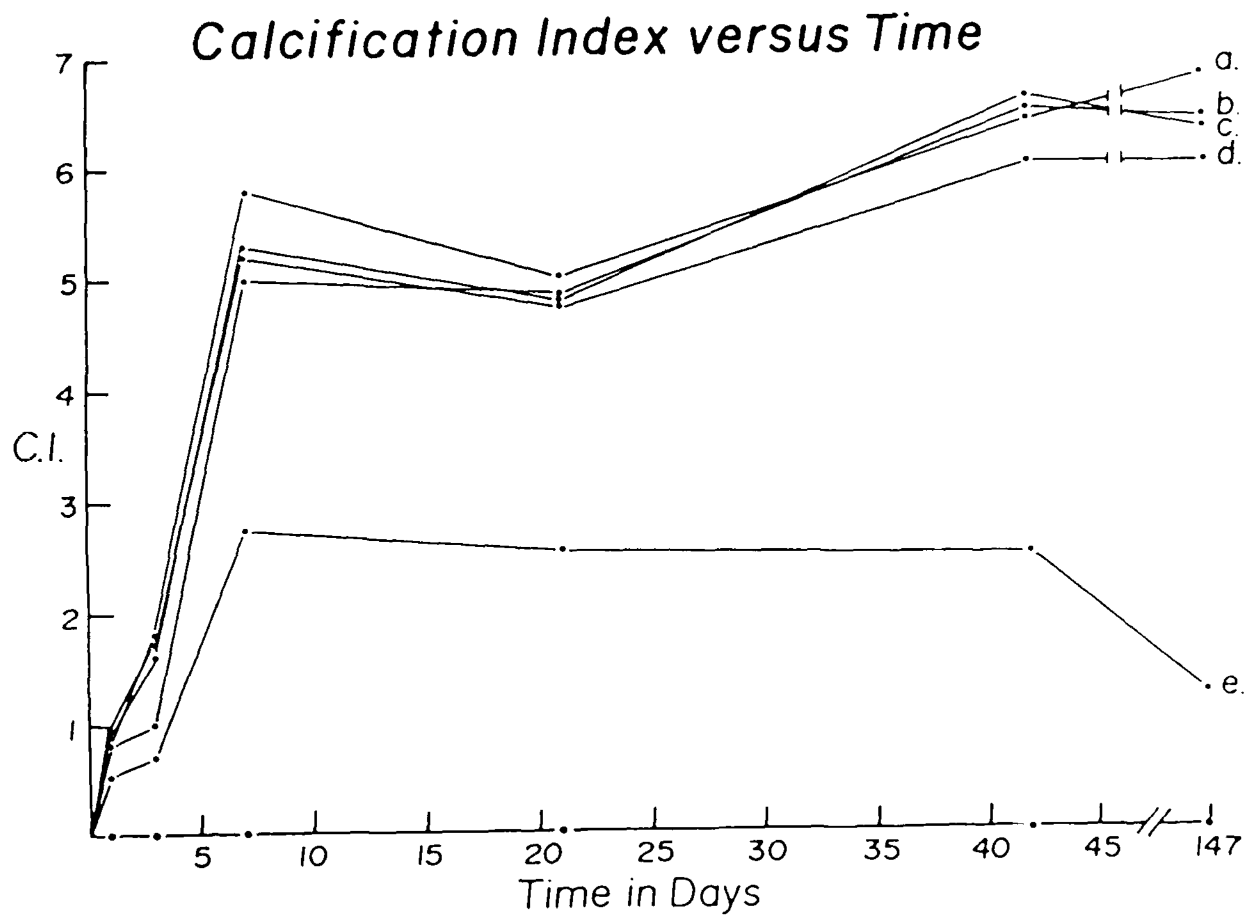


FIGURE 5

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