International Symposium of Research Students on Materials Science and Engineering
December 20-22, 2004, Chennai, India
Department of Metallurgical and Materials Engineering, Indian Institute of Technology Madras

MINERALISATION AND CROSS-LINKING TECHNIQUES TO IMPROVE THE MECHANICAL PROPERTIES OF COLLAGEN-CALCIUM PHOSPHATE (COLL-CAP) COMPOSITES TO BE USED AS BONE ANALOGUES.

D. Gotora and JT.Czernuszka
Department of Materials, University of Oxford, Oxford, United Kingdom

ABSTRACT

Bone is subjected to such widely varying stress conditions that no one material can effectively handle them all. A composite material is nature's answer to this problem. Bone as a material can be thought of as a polymer-ceramic composite, made up of a fibrous protein matrix (collagen) that is stiffened by a mineral phase (carbonated hydroxyapatite) with water acting as a plasticizer and porosity/matrix structure essential for nutrient transport and mechanical response. Trying to engineer an adequate replacement for bone - fulfilling all the roles of natural bone - is a tall order. However an adequate temporary replacement, whose primary role is that of support, is within grasp.Collagen-Calcium Phosphate composites (Coll-CaP) are novel materials that have the potential to be used as bone analogues. These composites can be made in a number of ways we produce Coll-CaP composites by the precipitation of calcium phosphate from aqueous solution into a collagen matrix. Next we strengthened the composite by a combination of cross-linking techniques together with mineralisation. We investigated the possibility of a superposition additive effect by different strengthening techniques combinations. From our experiments we observed that the mineral phase (30-40 % weight percent in the composite) significantly stiffened the collagen matrix. The introduction of artificial cross-links using the agents Diphenyl phosphoryl azide (DPPA) and Glutaraldehyde (GTA) into the composite before precipitation, further improved the composites Young's Modulus to 1.30 GPa (Dry).

1.0 INTRODUCTION

Strengthening of soft or hard tissue replacement materials by cross-linking techniques and mineralisation is well documented ((Iijima, Moriwaki et al. 1994; Kikuchi, Itoh et al. 2001). There has been fair success in soft tissue replacements (Li 2000), however generally hard tissue substitute materials fail to match host hard tissue mechanical properties ((Mathers and Czernuszka 1991; Lawson and Czernuszka 1998)

This paper describes the investigation carried out to improve Collagen-CaP stiffness by combining mineralisation and cross-linking strengthening techniques. The effect of cross-linking treatments on mineral precipitation into the matrix, mineral distribution and mineral phase type is investigated with appropriate techniques. Finally the complimentary and competitive nature of combined cross-linking and mineralisation is described and discussed in regards to the strengthening of collagen matrices of varying architectures.

2.0 MATERIALS AND METHODS

Collagen matrices of varying architecture were subjected to combined cross-linking treatments and mineralisation techniques. These strengthening techniques are summarised in Table 1.

Matrices that underwent crosslinking treatment followed by biomimetic precipitation were referred to as XP samples, while those that were first subjected to the precipitation procedure before cross-linking as PX samples. The suffix (e.g. GTA or DPPA) following either one of these sample tags indicates the crosslinking agent used in the cross-linking treatment stage.

GTA is a bi-functional reagent that reacts with the ε-amino groups of lysine residues. The GTA molecule itself forms the cross-link between the amino groups on adjacent chains, hence resulting in an intermolecular cross-link. As the GTA concentration is decreased intra molecular links are observed. Instead of adding a bi-functional cross-link between collagen molecules (as in GTA treatment), this DPPA treatment processes the carboxylic functional groups of aspartic and glutamic amino acids on collagen molecule such that they react with each other to form urea cross-links (Khor 1997). The transformation of carboxylic acid groups into acryl azide groups is followed by one of two reactions – either reaction with amino acid of an enzyme or with a molecule on an adjacent collagen molecule (Petite, Frei et al. 1994).

3.0 RESULTS

The combination of cross-linking followed by mineralisation produced stiffer composites in comparison to the same matrices untreated and separately cross-linked or mineralised.

For matrices of varying thickness the multilayered matrices were generally seen to have significantly higher stiffness than single layered matrices (Table 2). Looking at the multilayered samples the stiffness of XP-GTA composites is seen to decrease with an increase in thickness, while that of XP-DPPA composites remains approximately constant (Figure 1). Amongst the multilayered composites the highest Young's modulus value (1170 MPa) was obtained for the I-2 matrix treated with GTA before mineralisation. This value however did not significantly differ from those determined for XP-DPPA I-2, I-4, I-8 or II-1 matrices (Table 2).

With regards to composites of varying collagen content it was seen that matrices of higher collagen content possessed higher stiffness values than those with a lower collagen content (Figure 1 B). The stiffening effect of the combined cross-linking and mineralisation treatments in XP-GTA composites is greater in 1 wt% matrices than 2 wt % matrices, but noticeably lower in multilayered 2 wt% matrices than in XP-DPPA composites. Both XP-GTA and XP-DPPA had significantly higher stiffness values (1021 and 1026 MPa respectively) for II-1 matrices compared to I-1 matrices (443 and 584 MPa respectively). Of all the matrix architectures the highest modulus value (1380 MPa) was measured for a double layered 2 weight percent collagen matrix (II-2) treated with DPPA before mineralisation.

4.0 DISCUSSION

Separately mineralisation and cross-linking increased the stiffness of collagen matrices, however though the combination of these two strengthening mechanisms did increase the stiffness it was not in the additive way that was hypothesised earlier.

In thin I-1 matrix cross-linking is the dominant strengthening mechanism as mineralisation is observed not to further increase the matrix stiffness (Table 3). This can be attributed to the

competition for cross-linking and nucleation sites as discussed earlier in structures with limited collagen bulk available for cross-linking and mineralisation.

In thick matrices (I-4, I-8 and II-2) the poor permeability of GTA cross-linked matrices due to the tanning effect and also the matrix density effect results in low mineral content for re-enforcement. Therefore little or no increase in stiffness due to the combined cross-linking and mineralisation was observed (Table 3). DPPA treated matrices however showed favourable increase in stiffness after mineralisation of these thick matrices. This is credited to the better permeability of DPPA treated matrices and therefore enable mineral to nucleate deep in the bulk of matrix. The complimentary effect of combined mineralisation and cross-linking thus further re-enforces the structure and therefore stiffens composite.

As a result of the highest results were achieved for the thick XP-DPPA II-2 matrix whose Young modulus rose by 18 % to 1318 MPa. The stiffness of II-1 matrices however was not far of as XP-GTA and XP-DPPA matrices were found to have stiffness values of 1026 and 1021 MPa respectively.

Generally with regard to XP-GTA matrices the cross-linking effect is the dominant strengthening mechanism while for XP-DPPA matrices the complimentary nature of combined cross-linking and mineralisation results in greater stiffening. High percentage increases in I-2 and II-1 XP-GTA matrices can possibly be explained by the electrochemical and stereochemical distribution of long polymerised GTA cross-links providing more potential nucleation sites, resulting in further mineral filler re-enforcement after mineralisation (Mann, Webb et al. 1989)

The determination of matrix stiffness showed that XP-GTA treatment was more effective in thin or less dense samples, in which agent penetration and ion transport is easier. For more dense or thicker samples XP-DPPA treatment generally produced matrices of higher stiffness (Figure 1 and Table 3).

5.0 SUMMARY

Pre-crosslinked mineralised matrices are observed to have mineral weight content (≈37%) and phase (hydroxyapatite) similar to that of uncross-linked mineralised matrices. However the presence of a higher cross-linking density does have an affect on the precipitation rate as GTA and DPPA treated matrices are observed to have slower precipitation rates than un-treated matrices. This is most evident in GTA treated matrices whose ‘tanning’ effect greatly reduces matrix permeability. Nevertheless the sequence of cross-linking before mineralisation (XP) was shown to be the better of the two as favourable mineral content and cross-linking densities was achieved via this sequence.

Though the hypothesis that; ‘the combined cross-linking and mineralisation obey an additive superposition principle’ must be rejected, the combination of crosslinking and mineralisation was observed to have significant effect on the matrix Young’s moduli. In comparison to pure collagen matrices combined cross-linking and mineralisation resulted in stiffness increase of up to 88 % in the thin matrices and 43 % in thick and denser matrices. The highest Young’s moduli results achieved by combined cross-linking and mineralisation were for XP-DPPA 2 wt % composites (1.0 GPa for II-1 and 1.3 GPa for II-2) and XP-GTA double layered 1 wt% composites (1.1 GPa).

REFERENCES

Iijima, M., Y. Moriwaki, et al. (1994). "In Vitro Crystal Growth Of Octacalcium Phosphate Crystals On Type I Collagen." Journal of Crystal Growth 137: 553-560.

Khor, E. (1997). "Methods for the treatment of collagenous tissues for bioprostheses." Biomaterials 18: 95 - 105.

Kikuchi, M., H. Itoh, et al. (2001). "Self-organisation mechanism in a bone-like hydroxyapatite/collagen nanocomposite synthesized in vitro and its biological reaction in vivo." Biomaterials 22: 1705-1711.

Lawson, A. C. and J. Czernuszka (1998). Proceedings of the Institute of Mechanical Engineers Part H: Journal of Engineering in Medicine 212: 413-425.

Li, S.-T. (2000). Biologic Biomaterials: Tissue derived biomaterials. The biomedical engineering handbook. J. D. Bronzino. Boca Raton, Fla., CRC Press published in cooperation with IEEE Press: 2 v.

Mann, S., J. Webb, et al. (1989). Biomineralisation : Chemical and Biochemical Perspectives. Weinheim, V .

Mathers, N. and J. Czernuszka (1991). "Growth of hydroxyapatite on type 1 collagen." Journal of Material Science Letters 10: 992-993.

Petite, H., V. Frei, et al. (1994). "Use of Di-phenyl-phosphoryl-azide for cross-linking collagen-based biomaterials." Journal of Biomedical Materials Research 28: 159 - 165.

TABLES

Table 1: Summary of the cross-linking treatments and the biomimetic precipitation method used for the combined cross-linking and mineralisation experiments.

	X	P
Strengthening Technique	Cross-linking Treatment	Biomimetic Precipitation
	GTA – treat matrix in 0.5% GTA, 24hrs, rt, wash in distilled water 24 hrs Or DPPA – treat matrix in 0.5% DPPA in DMF, 24hrs, 4 C, wash in borate buffer, & water	Precipitate HAp into matrix from pH and temperature controlled solution at -: pH – 8 Temp – 37 C Stirred at 3Hz 14 hours

Table 2: Young's Moduli of pre-cross-linked mineralised composites

Sample	XP-DPPA	XP-GTA
Varying thickness	Young's Moduli in MPa	
I-1	443 {*a*}	584 {*a*}
I-2	1052 {*b*}	1170 {*b*}
I-4	1059 {*b*}	988 {*c*}
I-8	1118 {*b*}	912 {*c*}
Varying collagen content		
1 Wt % (I-1)	443 {*a*}	584 {*a*}
2 Wt % (II-1)	1021 {*b*}	1026 {*b*}
Moduli followed by the same letter do not significantly differ		

Table 3: Table showing the percentage increase of the stiffness of cross-linked collagen matrices, after mineralisation

	E (MPa) for Cross-linked Matrices		Strengthening Techniques Percentage Increase	
	X-GTA	X-DPPA	XP-GTA	XP-DPPA
I-1	751	786	0%	0%
I-2	693	762	69%	38%
I-4	903	748	9%	41%
I-8	749	771	22%	45%
II-1	716	999	43%	2%
II-2	1065	1115	0%	18%

FIGURES

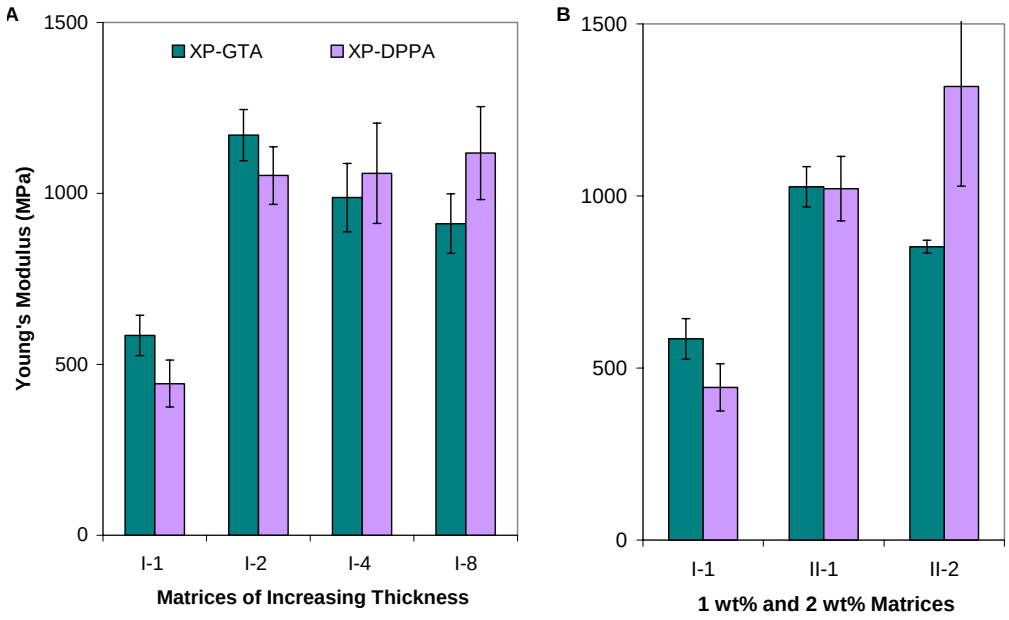

Figure 1: Graphs comparing the Young's Modulus of pre-crosslinked mineralised matrices of different (A) Thickness and (B) Collagen content. (All mechanical testing are performed on a Perkin-Elmer Mechanical Analyser DMA 7e under extension in static loading)