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Preliminary work on the development of a novel detection method for osteoporosis

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Abstract Osteoporosis affects both the organic and mineral phases of bone resulting in a decrease in resistance to fracture. Dual x-ray absorptiometry (DEXA) scans are used for diagnosing osteoporosis, which is conventionally characterised by a decrease in mineral density. Unfortunately, some patients who suffer osteoporotic fractures have normal bone density, because both the organic and the mineral phase are affected. However, there are currently no methods of evaluating the health of the organic phase. Patients undergoing treatment for osteoporosis have reported hardening of their fingernails. As the properties of nail and bone may be linked in a comparable, measurable way, this work used both mechanical (nano-indentation) and chemical (Raman spectroscopy) methods to evaluate differences between fingernails sourced from osteoporotic and non-osteoporotic patients. The difference in mean modulus between the nails sourced from the groups was 1.1 GPa. The disulphide bond content of fingernail samples from each group was measured by Raman spectroscopy and disulphide bond content of fingernail was found to be significantly lower in the osteoporotic group. It can be concluded that a relationship between the mechanical and chemical properties of nail and bone may exist in a measurable way. This work has suggested that changes in the organic phase of bone are reflected in similar proteins, such as keratin, from which fingernails are composed. Collagen and keratin are two distinct structural proteins, but they share the need for protein sulphation and disulphide bond formation, via cysteine, for their structural integrity. A disorder of either process should lead to disordered collagen and keratin synthesis.

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1 Introduction

Osteoporosis is a bone disease characterised by a deficiency of bone affecting both the protein matrix and the mineral fraction resulting in a decrease in the resistance of bones to fracture. The current method of diagnosis is dual x-ray absorptiometry (DEXA), which provides a quantitative measurement of the amount of mineral present. A decrease in bone mineral density (BMD) as measured by DEXA, is the current method of diagnosing osteoporosis and predicting fractures [1, 2]. However, there is overlap in the BMD between individuals who have sustained fractures and those who have not, suggesting that low BMD is not the only cause of fragile bones [3]. Thus, while the degree of mineralisation is the current standard by which osteoporosis is diagnosed, it is unable to detect bone fragility due to deficiency in protein matrix.

Bone is a composite material, comprising mineral, organic, and water phases [4]. The mineral phase, mainly hydroxyapatite (HA), imparts compressive strength, while the organic phase, collagen, imparts flexibility. Wang et al. [5] have shown that with increasing age, the fracture toughness of bone is decreased and its microhardness increased without significant changes in BMD. McCalden et al. [6] reported similar findings, indicating that even without significant changes in BMD, the tensile strength of bone can decrease with age due to increased porosity. There is now a belief that the organic phase of bone plays a significant role in osteoporosis. Kovach et al. [7] have shown that changes in the structural characteristics of the collagen network detected using a laser fluorescence technique correlate significantly with bone fracture toughness. This work is backed up by other findings [8, 9] demonstrating that the organic phase of bone is responsible for much of its ability to resist fracture. Mansell and Bailey [10] found that collagen in osteoporotic bone was not normal, but contained higher levels of lysine hydroxylation and modified cross-linking. This and other studies [11, 12] concluded that osteoporosis had a degenerative effect on protein production in bones with increased immature collagen cross-links, increased collagen synthesis and degradation (increased turnover despite overall loss of collagen) as well as reduced mineralisation. The increased hydroxylation leads to the formation of finer fibrils with altered crosslinks, and reduced calcification, which further contributes to the fragility of the bone.

Having established that osteoporosis is a disease which affects the protein phase of osseous tissue it is reasonable to expect that changes in one fibrous structural protein, collagen, will be reflected in similar proteins, such as keratin, from which fingernails are composed. Hence, relating degenerative changes in the mechanical properties of bones to analogous changes in the properties of fingernails formed the basis of this research.

Keratin molecules are helical and fibrous, twisting around each other to form strands called intermediate filaments. These proteins contain a high percentage of sulfur-containing amino acids, largely cysteine, which form disulphide bridges between the individual molecules. Disulphide bridges, or sulphur to sulphur bonding of two cysteine residues, function to link together the various peptide constituents of all proteins. Disulphide bridges also aid in the maintenance of structural rigidity in keratin protein. ''Hard'' keratin, as is found in hair and nails, has a greater amount of structural rigidity due to more disulphide bonds.

Fingernail examination has potential as a screening method for osteoporosis. Previous work in this area includes a study which compared calcium and magnesium levels in bone with those in nails [13]. No correlation was found to exist between the two. Despite the fact that taking the same measurements from nail as are taken from bone is perhaps intuitive, expecting to see Ca or Mg levels indicative of bone was unlikely to succeed since the mineralisation process in bone is absent in the fingernail. Other research into finding a relationship between mineral concentrations in nail and bone have taken a broader approach and have suggested that significant correlations exist between zinc levels and BMD ($r = -0.399$) and between the ratio of Zn/Ca to BMD ($r = 0.421$) [14]. These findings are of importance in demonstrating how the composition of nails are influenced by skeletal changes, and demonstrate the potential for using nail tissue in the diagnosis of systemic disease.

In this study the results of the fingernail tests were compared only to BMD readings for each volunteer. Since mineralisation does not occur in fingernails, for this comparison to have significance two conditions must be met.

1. There is a recognised decrease in the quality of the protein produced in osteoporotic bone
2. It is this deterioration that damages the mineralisation process or that the decrease in mineralisation correlates with a deterioration in collagen, both being affected by the disease.

Since it has been previously shown that the type 1 collagen in bone is adversely affected by osteoporosis, only the second condition mentioned above remains to be addressed, namely, that decreases in mineralisation and protein quality are interrelated. This link is crucial since this research sought to find a relationship between keratin and bone collagen by comparing keratin and bone mineralisation, since mineral densitometry is the current means of distinguishing between osteoporotic and non-osteoporotic bones.

Studies have shown that type 1 collagen arranged into fibrils can support apatite formation from calcium phosphate solutions [15] and that other bone matrix proteins help control the bone mineralisation process, the most effective of these being bone sialoprotein (BSP) [16]. It is also known that the extent of the damage to protein is dependent on the severity of the disease, as is the degree of mineralisation, suggesting that as one declines so does the other. The exact nature of this relationship is unknown however, except to say that it is not simply a linear relationship, as indicated by the overlap in BMD between bones that fracture

and ones that do not. This implies that a straightforward linear relationship is unlikely to be found between keratin and BMD either, although it does show that a relationship should exist. Therefore if the degree of mineralisation is known and is in some way correlated to collagen breakdown, and the process that causes this also adversely affects the structural rigidity of bonded keratin, it is not unreasonable to attempt to find a correlation between fingernail properties and BMD. The human nail plate is one of the most impervious biological structures and the penetration of chemical agents into it is low. However, its physical properties change when soaked in water, as it becomes soft and flexible. It is thought that the degree of hydration is the most important factor influencing the physical properties of nails[17] as chemically bound water is found in both dry and wet nails, indicating water protein interaction [18] and this interaction changes the keratin structure giving it new mechanical characteristics. This highlights the need to keep volunteer samples in conditions where they are not exposed to large amounts of water, or dehydrated and so prior to testing a protocol had been developed that established suitable storage and testing conditions for the nails.

2 Methods

2.1 Clinical sourcing of nails

Two groups of subjects were identified. The first group (n = 9) were diagnosed by DEXA (Lunar Prodigy, GE Medical systems), as osteoporotic (T score < –2.5). The second group (n = 13) were non-osteoporotic (T score > 1.0).

Fingernail clippings were obtained from all subjects. The nail apparatus is composed of the nail fold, nail matrix, nail bed and the hyponychium, which together form the nail plate. This nail plate is produced mainly by the matrix and emerges via the proximal nail fold, while being held in place by the lateral nail fold. It overlays the nail bed and detaches at the point called the hyponychium, or where the free edge of the plate ends. This is where the clipping is taken (Fig. 1). This area corresponds to the area where high sulphur keratin, typical of hard keratins, is found. Following their sourcing, samples were stored in sealed specimen jars prior to testing.

2.2 Nano-indentation

The nails were trimmed prior to testing to expose the flat mid-section of each nail, and, therefore, reduce the possibility of the curved edges of the nail making premature contact with the indenter. Samples were then attached to aluminium stubs with an epoxy adhesive (part no: 46409, Versachem, FL, USA).

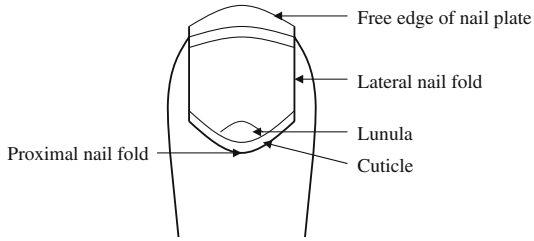

Fig. 1 schematic of fingernail. The nail clippling is taken from the free edge of the nail plate

Nanoindentation experiments were conducted using a laboratory-built machine previously described by Arteaga et al. [19]. For each indentation the tip was brought into contact with the surface using a load of a few μN. The load was then increased linearly at 0.8 m Ns^{-1} up to its maximum value of 120 mN, and then reduced again at the same rate to zero. Every 150 ms during the cycle readings were taken of the displacement of the indenter δ, and the load P, allowing the examination of force-penetration data during both the loading and unloading phases. Consequently, it is possible to produce curves of penetration depth at each force level during the loading and unloading phases. A schematic representation of a nanoindentation curve is given in Fig. 2.

Modulus and hardness were identified as potential indicators of nail brittleness. The modulus was calculated from the linear section of the unloading curve (b,c in Fig. 2).

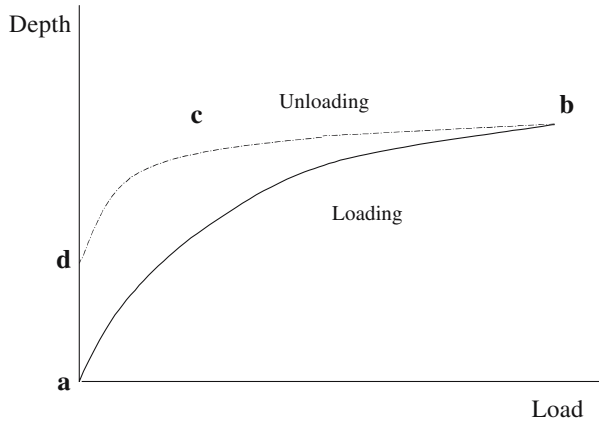

Fig. 2 Schematic representation of a typical nanoindentation curve with loading (a,b), and unloading (b–d) phases

Hardness, H, is defined as

$$H = \frac{P}{A} \quad (1)$$

where P is the force applied to the indenter and A is the projected area of the contact. In nanoindentation the projected area of contact is calculated from the geometry of the indenter and the measured depth of penetration in contact with the indenter, h, using [31],

$$A = k h^2 \quad (2)$$

where k is a constant dependant upon the geometry and type of indenter used. In this study, the indenter was a trigonal diamond pyramid with an equilateral triangular cross-section and a 90° angle between each face and the opposing edge (the corner of a cube). For this indenter $k = 2.6$. Substituting (2) into (1) gives:

$$H = \frac{P}{2.6 \bullet \delta_p^2} \quad (3)$$

While the indenter is moving into the material the load P has to provide the stress field, which is necessary to support the plastic flow of material out of the indentation as well as the static pressure equal to the hardness. Due to this, the curve of dynamic hardness as a function of depth derived from (1) usually has a very high value at small depths where the strain rate, which is proportional to$1/\delta$ dδ/dt, is greatest. A single hardness number is quoted from the results and this is taken as the maximum applied force where the strain rate is a minimum.

2.3 Raman spectroscopy

For Raman analysis, four fingernail samples from each group were analysed to ascertain if there was disparity between groups, and to detect osteoporotic induced changes in keratotic tissue. Micro Raman spectra were obtained using a Dilor Labram 01 instrument. Excitation was by red laser operating at 632.81 nm. Spectra were obtained by performing 20 scans, to improve the signal-to-noise ratio, each with a laser exposure time of 50 s. The same operating procedure was repeated for all samples in order for the resultant spectra to show only the differences between the osteoporotic and non-osteoporotic tissue. Spectra were recorded from 300 cm^{-1} to 1800 cm^{-1} for identification of all the characteristic peaks in human nail. The interval from 300 cm^{-1} to 700 cm^{-1} was selected for comparison. Normalisation of all acquired spectra was carried out to facilitate the comparison, and to highlight differences between groups.

3 Results and discussion

3.1 Nano-indentation

Mean elastic moduli and hardness results for the two sets of fingernails are included below in tabulated (Table 1) form.

The mean moduli of fingernails from patients with low BMD are approximately 25% lower than those with normal BMD. The difference in mean modulus between the groups was found to be 1.1 GPa but this was not significant at the 5% level ($p = 0.147$).

3.2 Raman spectroscopy

Figure 3 shows the typical Raman spectrum of human nail between 300 cm^{-1} and 1800 cm^{-1}. All the major spectral peaks of human nail identified previously [20, 21] were found. These include the amide band at 1677 cm^{-1} indicating that nail keratin is predominantly α-helical, the methylene (CH_2) deformation band at 1450 cm^{-1} and the amide [ν(CN)] band at 1251 cm^{-1}. In the 1000 cm^{-1} to 1200 cm^{-1} region the strongest band occurs at 1006 cm^{-1}, corresponding to the C-C stretching vibration of the aromatic ring in the phenylalanine side chain. However it is the lower region of the spectrum that is of most concern in this study. The area between 700 cm^{-1} and 300 cm^{-1} contains the spectral information about the sulphur bonding in fingernails. The relative intensities of the S-S and C-S stretching vibrations give a good indication of the amount of sulphur present and allow determination of the structural configuration of the S-S bond. Figure 3 shows the peak at 510 cm^{-1} representing the disulphide bonding [ν (SS)]. Lesser peaks at 621 cm^{-1} and 645 cm^{-1} represent carbon sulphide bonding [ν (CS)].

Figure 4 shows normalised Raman spectra for an osteoporotic and non-osteoporotic nail on the same scale. From the results of this work two main differences were seen to exist between the two groups of nails.

Table 1 Mean moduli and hardness (and standard deviations) of fingernails sourced

Subject Group	Moduli (GPa)	Hardness (GPa)
Osteoporotic	3.0 (±1.5)	0.19 (± 0.12)
Healthy	4.1 (± 1.1)	0.23 (± 0.14)

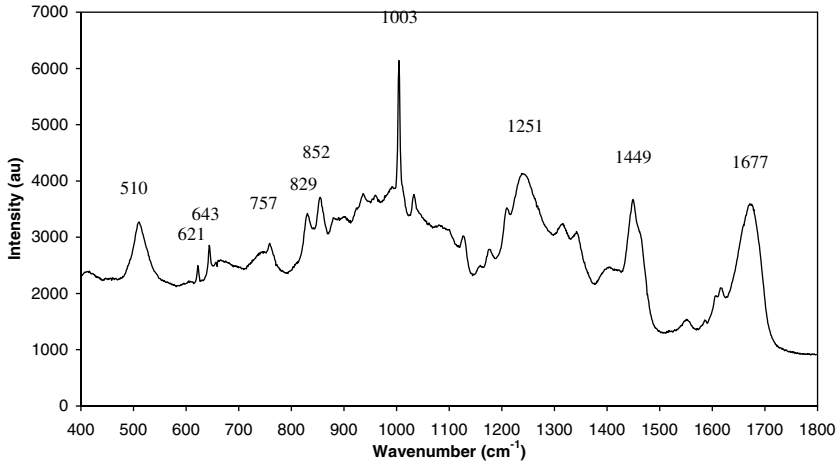

Fig. 3 Human Nail Raman Spectrum. 300 cm^{-1}–1800cm^{-1}

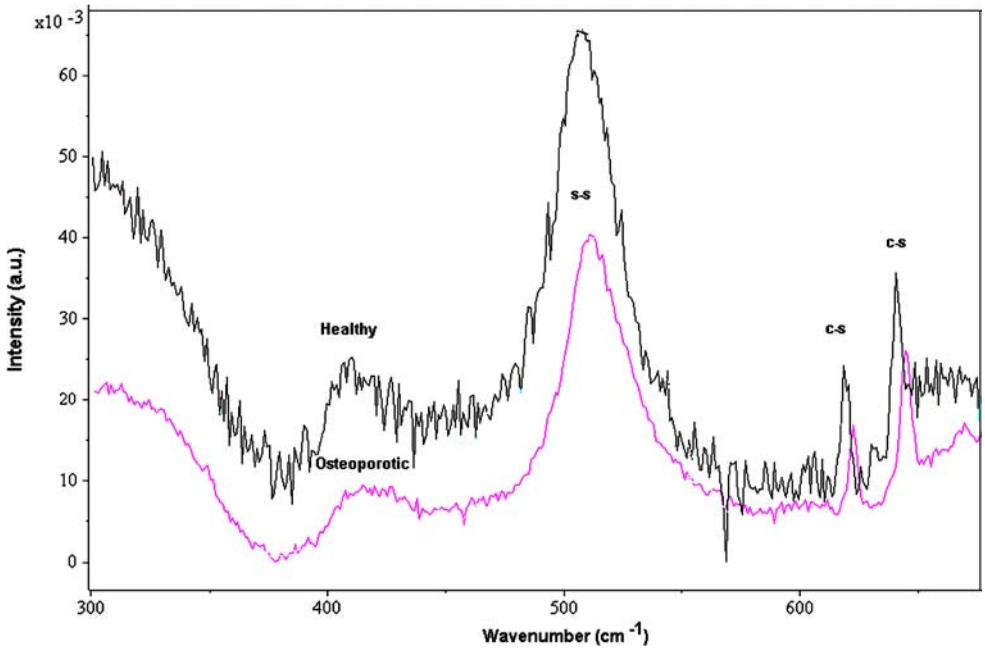

Fig. 4 Raman Spectra from Non-Osteoporotic (top) and Osteoporotic (bottom)

1. The disulphide bond (S-S, *gauche-gauche-gauche* conformation) peak for healthy nail at 510 cm^{-1} was much sharper than for the osteoporotic nail and the width of the S-S peak in osteoporotic nail was found to be larger than the healthy nail. Therefore, the disulphide bond content of the nails sourced from osteoporotic patients was lower than those from healthy patients. Table 2 shows that this difference in mean width at half maxima for the S-S peak from the two sets of nails is statistically significant but this will need to be repeated on larger sample sizes for further conclusions to be drawn.
2. There was also a shift in the carbon sulphide bond (C-S) peak at about 621 cm^{-1} and 643 cm^{-1} as shown by the higher wave numbers detected for the C-S bonds in osteoporotic nail. The reason for this is not clear. This may be due to the change of the sulphur content in the nails since it is known that the C-S stretching vibration is dependent on the conformation of its side chains.

Table 2 Raman spectroscopy results for osteoporotic versus non-osteoporotic nail

Width at half maxima for the S-S peak	(cm^{-1}) Minimum	(cm^{-1}) Maximum	(cm^{-1}) Mean	Std. Deviation
Non-osteoporotic	25.00	30.70	27.68	2.39
Osteoporotic	37.50	42.30	39.20	2.12

In protein spectra the C-S vibrational band originates from methionine, cysteine and cystine. Since

methionine content in human nail is negligible [22] the C-S and S-S bands shown must have originated from cysteine and cystine. This would fit the hypothesis of reduced cysteine or sulphur playing a role in nail brittleness and bone fragility. The reason for the shift in carbon sulphide bonding is not yet clear. This may be due to the change of the sulphur content in the nails since it is known that the C-S stretching vibration is dependent on the conformation of its side chains.

4 Conclusions

Although there were measurable differences in both the nano-indentation and spectroscopy results of osteoporotic versus non-osteoporotic patients, the differences in nanoindentation were not statistically significant. The reason for this may be that there was no co-efficient of variation for nano-indentation for human fingernails. Given the wide variation of results, the intra-assay co-efficient of variation may demand larger study numbers to ensure a statistically valid result.

The measurement of nail hardness, modulus and disulphide bond content should be further studied in sufficient numbers to detect a significant difference. The advantage of examining nail lies in the ability to assess properties other than those measured by DEXA, its accessibility and its more rapid growth, allowing change to be monitored on a more frequent basis. The validity of using total hip BMD to look at sequential results has been brought into question.[23] The development of a non-invasive method of measuring quality of bone, rather than bone mass, for screening or for follow-up of treated patients would be of considerable interest. The small scale of this study means that the suitability of nail testing as such a method is inconclusive but these preliminary findings suggest that changes in bone proteins seen in osteoporosis may be mirrored by changes in related structural proteins.

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