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Development of a sensitive whole blood chemiluminescence method for assessing the bioactivity of calcium phosphate powders

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Abstract

The development of simple, but highly sensitive and accurate in vitro methods for assessing the bioactivity of biomaterials is a key requirement for assuring their successful clinical application. Moreover, screening techniques that will allow various materials to be recommended, or otherwise, for further testing, have the potential to make appreciable savings in the time and expense associated with product development.

This paper gives details of a novel whole blood chemiluminescence method, based on the photoprotein Pholasin®, which emits light in the presence of the free radical superoxide, other reactive oxygen species and the enzyme myeloperoxidase. This whole blood assay may have the potential as a screening method for assessing calcium phosphate (Ca-P) bioceramic powders according to their relative bioactivity. The method employed involves exposure of samples of anti-coagulated (heparinised) blood to the various Ca-P materials and stimulating the contacted leukocytes in the blood with phorbol-12-myristate-13-acetate (PMA). PMA stimulates protein kinase C directly and sets in motion a cascade of events in the leukocytes leading to the activation of the superoxide generating NADPH oxidase system. PMA also promotes degranulation, which is the fusion of the granule membrane with the plasma membrane and the extracellular release of granule enzymes.

Changes in the free radical and degranulation activity of the cells, as measured by the luminescent response of Pholasin® to cells stimulated with PMA, after exposure to the Ca-P powders, are presented as percentage differences from the response to (control) blood samples not exposed to the powders. The resultant data clearly indicate significant differences in the light detected via Pholasin® from the blood exposed to the bioceramic materials compared to that from the relevant controls. These differences correlate well with the ascribed bioactivity of the Ca-P powders, as assigned from the measured chemical and structural properties. Furthermore, the method can also be applied to samples of blood, which have been diluted in the ratio 1:100 in standard blood dilution buffer. The light output response in the diluted blood experiments is exactly the same as that observed with undiluted whole blood but occurs immediately as compared to the many minutes required to give response in the latter case. Thermally processed (800°C) Ca-P materials have also been studied. A relationship between the heat-induced properties of the powders and the chemiluminescence responses recorded from blood cells stimulated with PMA has been established. © 2002 Published by Elsevier Science Ltd.

Keywords: Bioactivity screening; Calcium phosphate powders; Leucocytes; Chemiluminescence; Free radicals; Pholasin®

1. Introduction

The materials used to fabricate medical or dental implant devices are, by necessity, required to be compatible with any host biological systems that they come into contact with. This inherent, or in some cases invoked, biocompatibility is critical to their long term performance and the subsequent device survival in vivo. As such, in vitro tests, which can provide an accurate measure of this important property, are a mandatory part of successful product development. Much of the recent emphasis in biomaterials research has been directed toward the provision of bioactive systems which have the potential to interact in some positive

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manner with host tissue so as to initiate cellular repair and, ultimately invoke tissue regeneration [1]. As such, these types of biomaterials require more exact test procedures in order to realise their full potential and, importantly, for their active properties to be predicted and controlled.

Clearly, a means of accurately and reliably determining how changes in the composition and structure of such biomaterials influences, or otherwise, any interaction with a biological system is of utmost importance. Moreover, screening techniques that can identify those materials which might have properties that warrant more detailed (and expensive) biocompatibility testing to be carried out, would play a valuable role in enhancing product development. Currently, the method of choice in this regard for bioactive ceramic materials is the relative "speed" with which a material deposits a layer of carbonated hydroxyapatite (CHA) from solution when exposed to simulated body fluid (SBF) [2–5]. However, this approach is invariably a slow process with even the most reactive of materials requiring several days to produce the required result to a readily measurable extent. In addition, the technique is not quantifiable in that the response is either positive or negative. Hence, provision of a method that is fast, easy to use, reproducible and of relevance to the biological environment in which the material will eventually be implanted has obvious benefits.

A convenient and relevant approach to such testing would be to expose the material, in the bulk form, to blood and to then assess whether the white blood cells have been affected by any chemical interaction. If successful, this approach could include an automated sample handling system in which the materials are placed within the wells of a microplate and exposed to blood for a pre-determined time period and aliquots removed for stimulation and subsequent analysis in a high throughput microplate reader luminometer. This of course assumes that such exposure can lead directly to activation of the cells or, at least, to their priming (both up and down-regulating), which could then be measurable and, moreover, quantifiable. Such interactions are normally desirable in an implant context, especially where they involve a reaction with bone and any attendant soft tissues surrounding an implant site, in a planned and predictable manner. Other interactions can, however, lead to an undesirable inflammatory response when, for example, the material may affect the activity of white blood cells or cause these cells to give abnormal responses to stimuli at a later time [6]. It is suggested that this type of activation could lead to the production of free radicals and the expression of adhesion molecules, as well as the production of chemicals such as, cytokines, chemokines, etc. [7]. These species could then activate other leucocytes and signal their recruitment in vast numbers to the site of the inflammation, represented by the implanted material. However, as noted earlier, exposure might not actually activate the cells but instead cause them to respond abnormally at some later time. Obviously, it is important to attempt to distinguish between these two possibilities.

Calcium phosphate bioceramics are an important class of inherently bioactive materials, which are used in a range of medical (mostly orthopaedic) and dental implant devices [8–10]. Hence, they lend themselves to act as model system for the development of screening techniques to assess the nature and scale of this bioactivity. The most widely used of these materials is hydroxyapatite (HA, $(Ca_{10}(PO_4)_6(OH)_2)$. There are several commercially available grades of HA and related compounds, which have been developed specifically for clinical applications. In addition, there are several relatively simple synthetic routes to obtain Ca-P materials. In particular, a microwave-assisted synthesis, which results in a high quality material, has been reported [11]. Due to its simplicity of operation, this technique offers a convenient way of producing a range of Ca-P phases, including stoichiometric HA. Tricalcium phosphate (TCP, $Ca_3(PO_4)_2$), is a form of Ca-P which is known to be clinically useful due to its positive response in vivo. Hence, TCP is normally classified as bioresorbable [12–14] rather than bioactive. However, for the purposes of this study it is deemed to be a highly reactive system and is used as a benchmark by which to judge the response of the other Ca-P powders. The chemical composition, crystallinity and stoichiometry (nominally the Ca/P ratio) of bioceramic materials are largely the factors that determine the degree to which they exhibit bioactivity in the bulk form [15,16]. These properties are greatly influenced by the nature of the source material as well as any processing conditions used to deliberately enhance key properties. For example, thermal conditioning is commonly used to enhance phase purity and/or the degree of crystallinity [17].

In this study, we report results from the application of a sensitive chemiluminescent whole blood test using the photoprotein Pholasin®, an ultra-sensitive detector of free radicals and degranulation enzymes [18–21] to monitor changes in leucocytes (mainly neutrophils) after their exposure to HA and TCP powders. Data are reported as the percent difference in the light output detected for the samples exposed to the various powders compared to that recorded for relevant controls. The effects exerted by thermal conditioning of these bioceramic systems at 800°C have also been determined and a similar comparative assessment of bioactivity obtained. Furthermore, the results presented have been used to probe whether exposure of the bioceramic materials to granulocytes in the blood leads to activation of the cells and/or if exposure of the powder to these granulocytes changes the way in which the cells

will subsequently respond to stimuli presented after exposure to these powders.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Superoxide and other reactive oxygen species (ROS) generated from stimulated blood cells were measured with a commercially available chemiluminescent assay kit used for determining the activation of leucocytes (predominantly neutrophils) from whole blood (ABEL® Whole Blood Test Kit, Knight Scientific Limited, Plymouth, UK). The test kit contained: Pholasin®, Adjuvant-P™, phorbol-12-myristate-13-acetate (PMA), assay buffer (HBSS with 20 mM HEPES, pH 7.4) and blood dilution buffer (HBSS without calcium and magnesium but with 20 mM HEPES, pH 7.4). The reagents were reconstituted with assay buffer to obtain stock solutions of: Pholasin® (10 μg ml^{-1}), Adjuvant-P™ (10 enhancement units ml^{-1}) and PMA (8 μmol ml^{-1}). Dulbeccos phosphate buffered saline pH 7.4 without calcium and magnesium (Sigma, UK) was used for the dispersion of the powders. Reagents used in the ABEL® Antioxidant Test for Superoxide (Knight Scientific Ltd, Plymouth, UK) were used to generate superoxide.

2.1.2. Calcium phosphate powders

The calcium phosphate (Ca-P) materials used in this study were: a commercial grade hydroxyapatite (HA, $Ca_5(PO_4)_3OH$, Merck KGaA, Germany); a Ca-P formulation, approximating to HA, synthesised by a microwave assisted method [11], hereafter denoted as HA(MW), and a commercial grade tricalcium phosphate powder (TCP, $Ca_3(PO_4)_2$, Fisher Scientific, UK).

In the case of the microwave-assisted method, the intention was to produce a stoichiometric hydroxyapatite (HA(MW)) with a Ca/P ratio of 1.67. Hence, a suspension of 0.24 mol l^{-1} $Ca(OH)_2$ in 200 ml of distilled water was stirred with 0.4 mol l^{-1} $(NH_4)HPO_4$ in 200 ml distilled water (total volume 400 ml). The reaction mixture was introduced into a 2.45 GHz microwave oven (Sanyo EM-S353) and irradiated at 900 W for 30 min. The rate of solvent "boil off" was checked at frequent intervals to avoid significant loss. After irradiation, the remaining solvent was removed by gentle heating on a hot plate for 30 min. The resultant precipitate was dried in an oven at 80°C for 60 min. Samples of each of the bioceramic materials were also heated in air at 800°C in a tube furnace (Carbolite, UK) for 2 h.

The particle size distribution for the six powders of interest was very similar, falling in the range 38–106 μm as measured by a Retsch Analytical Type AS200 Sieve Shaker. Hence, in relative terms, the associated active surface areas are deemed not to differ significantly. In addition, no gross differences in particle morphology were observed during SEM examination of the six powders.

2.2. Methods

2.2.1. Materials characterisation

The chemical composition and associated structural properties of both the commercial and laboratory synthesised Ca-P powders, before and after thermal conditioning, were determined by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD). FTIR analyses were carried out using a BIORAD Excalibur FTS 3000MX series instrument equipped with a Pike Technologies EasiDiff Diffuse Reflectance (DRIFTS) accessory. Spectra were recorded at a wave number resolution of 4 cm^{-1} in absorbance mode for the region 4000–400 cm^{-1} with 16 scans per sample. XRD of the powder samples was carried out using a Siemens D-5000 Diffractometer equipped with a Cu K_α X-ray source ($\lambda = 1.540$ Å) operating at 40 kV and 40 mA. Each diffraction scan was recorded from 2θ 3–63° with a step of 0.04° and a 1-s dwell time per increment. Measurement of Ca/P ratio in the various powders was made by energy dispersive X-ray (EDX) analysis using Oxford Instruments Link ISIS X-ray microanalysis system attached to a Hitachi S-3200N scanning electron microscope (SEM). All data were collected at an incident electron accelerating voltage of 20 kV. In the case of the unheated materials, the Ca/P ratio was confirmed by X-ray fluorescence (XRF) using a Spectro X-Lab 2000 ED-XRF. The powder samples were fused as glass disks prior to analyses using 1 g sample and 6 g of flux (lithium tetraborate). The fluxing was carried out using a Claisse Fluxer.

2.2.2. Collection of blood samples

Blood samples from five consenting adults were collected via venipuncture and placed into two individual preservative-free heparinised tubes (Becton Dickinson, Vacutainer systems, Meylon, France). The blood samples were stored at 4°C and used within 2 h of collection. Subjects enrolled in this research have given informed consent, which has been approved by a recognised institutional committee on human research. Two of the blood donors (A and E) were undergoing treatment for rheumatoid arthritis which involved taking medication at the time of donation. The other three donors (B, C and D) were then treated as normal controls within the accepted limitations of the size of the sample set.

2.2.3. Exposure of bioceramic powders to blood samples

The calcium phosphate materials used in these studies were not sterilised prior to exposure to blood from the five donors and the subsequent chemiluminescence assay. However, previous cell culture studies with these powders have indicated that the changes in the bioactivity of the various samples examined here is not contributed to by the destruction of residual contamination (e.g. lipopolysaccharide, LPS) as a function of heating.

Approximately 110 mg of each of the six different powders (three unheated and three heated) was dispersed in 10 ml of Dulbeccos phosphate buffered saline, pH 7.4, without the presence of calcium and magnesium. These stock suspensions were stored at 4°C until required for the assay. In all cases, control solutions, i.e. those which had not been exposed to the bioceramic powders, were prepared in the same way. After mixing, by vortex and inversion, 100 μl of each stock dispersion solution and the same volume of the PBS controls, were added to individual polypropylene tubes (with caps) containing 1 ml of each of the whole blood samples. The capped tubes were inverted six times and incubated at 37°C for 120 min. At various times over a total exposure period, 11 μl samples of blood plus the dispersed bioceramic powder were removed and added to 1 ml of blood dilution buffer. After mixing, 100 μl of the diluted blood plus powder was used directly in the chemiluminescence assay, as described below.

Dilutions of the stock materials were also made immediately before the diluted blood experiments. Accordingly, 100 μl of the stock powder suspension was added to 1 ml of blood dilution buffer and, after mixing, 11 μl aliquots of anticoagulated (and well mixed) whole blood, or 11 μl of PBS for corresponding controls, were added to create a 10 mg ml^{-1} suspension of powder in a 1:100 dilution of the whole blood samples. A 100 μl sample of this diluted blood plus powder suspension was used in the subsequent chemiluminescent assay.

This concentration was based on a method developed by Nagase et al. [22], who reported that values of 1 and 10 mg ml^{-1} of hydroxyapatite in blood were required in order to obtain a significant difference in the chemiluminescent response of neutrophils (in the presence of luminol). Hence, since Pholasin is more sensitive than luminol, the lower of the two concentrations of powder per ml of blood was used in these experiments.

2.2.4. Chemiluminescence assay and control procedures

For both the whole blood and diluted blood methods, 450 μl assay buffer, 250 μl Pholasin® (2.5 μg), 100 μl Adjuvant-P™ (1 enhancement unit) and 100 μl diluted blood plus bioceramic powder (or PBS for the associated control), were mixed together in a polypropylene luminometer cuvette and incubated in situ for 5 min at 37°C in a computer-controlled Bio-Orbit Model 1251 Luminometer (Bio-Orbit, Turku, Finland). The cuvette was then automatically moved to the measuring chamber and light output from the solution measured every second for a period of 90 s. At this point, 100 μl of PMA was injected into the cuvette, while still positioned in front of the light detector, to a final concentration of 0.8 mmol l^{-1}. Emitted light in relative units expressed as mV, was measured for a further 10 min while the cuvette was continually mixed. To determine the nature and magnitude of any possible changes to the blood samples over the duration of exposure, a control sample of the blood was assayed at the beginning and end of each set of experiments. Peak luminescent values were determined from a plot of light output (mV) versus time. The effect of exposing the various blood samples to the unheated and heated Ca-P materials was determined by calculating the mean percentage change in chemiluminescence output generated from the reactive oxygen species of stimulated blood cells, compared to that originating from the corresponding control samples.

All the assays reported here were run in duplicate but not with the same sample of blood in both cases. This is because we have learned over many such assays using whole blood that once a donor sample vial has been opened, it can show a marked change in the way it responds in any subsequent assay. For this reason two tubes of blood were collected from each of the five individual donors and an assay was carried out from blood from each tube as reported in Section 2.2.2.

2.2.5. Assessing possible quenching of light and/or free radicals by calcium phosphate powders

In order to assess the direct effect of the bioceramic systems on the quenching of light and/or free radicals, superoxide was generated in the presence and absence of the various Ca-P powders. Pholasin® (100 μl reconstituted antioxidant Pholasin®), 100 μl PBS or 100 μl suspension of powder and 600 μl of Solution A from the commercially available antioxidant kit for superoxide determination, were added to a cuvette. While the cuvette was in the measuring chamber of the luminometer, 100 μl of Solution B from the kit was injected. Superoxide is produced instantaneously with the addition of Solution B. The difference in peak luminescence measured from the cuvettes with powder, compared to that recorded for the corresponding controls, provides a measure of the scavenging of free radicals and/or quenching of light.

2.2.6. Determination of neutrophils viability

The viability of isolated neutrophils was determined before and after exposure to the various bioceramic powders as a means of assessing blood cell membrane integrity after contact. The method used was a standard trypan blue exclusion assay. The PMN leucocytes were isolated by density gradient centrifugation over Histo-

paque™ 1119 and 1077 g l^{-1} media (Sigma, UK). The PMN layer was aspirated and the cells collected washed twice with blood dilution buffer and re-suspended in the standard assay buffer. A 100 μl aliquot of cells was added to 100 μl of the buffer plus powder suspension and the solution incubated at 37°C for 20 min. A control solution comprising the cells and the same volume of PBS was also incubated in the same manner. For the trypan blue assay, 100 μl of the test and control solutions were added to 200 μl of 0.1% trypan blue (w/v) in 0.85% NaCl (w/v) and incubated for 3 min at 37°C. An aliquot of each solution was then placed in a haemocytometer and the cells examined under the microscope. Uptake of trypan blue was taken as an indication of death. Percent viability = number of viable cells (unstained)/number of viable + number of dead cells (stained) × 100. Percent viability of cells in the presence and absence of powder was compared. Whereas, it is accepted that it would have been preferable to assess the viability of the cells directly from whole blood samples, this was not possible here. Nonetheless, this approach does confirm neutrophil viability post-exposure to the assay conditions.

3. Results

3.1. Chemiluminescence of whole blood after exposure to tricalcium phosphate

As indicated earlier, tricalcium phosphate (TCP) is a highly reactive Ca-P bioceramic that is readily resorbed in vivo [12–14]. Hence, it was chosen here as a positive control for the in vitro tests on the materials of interest. In this way, it is envisaged that, if the test proves to be useful in assessing subtle changes in the chemical composition of novel bioceramic materials, then the response of white blood cells (neutrophils in whole blood and macrophages in tissues) exposed to such materials may be expressed in relation to a standardised response to TCP.

A set of luminescence response curves plotted as light emitted per second from the assay solution versus elapsed time in minutes are shown in Fig. 1. The light output occurs as a result of the interaction of free radical superoxide and degranulation enzymes with the photoprotein Pholasin® after cells from the heparinised blood (mainly neutrophils) have been deliberately stimulated with PMA. It should be noted that neutrophils are seldom activated while circulating in peripheral blood. Evidence of such activation in this test would therefore be revealed by a relatively high emission of light prior to injection of the stimulant coupled to a poor luminescent response after injection of PMA. As can be clearly seen from Fig. 1 none of the cells of the blood samples used here show any pre-stimulation activation.

The bioactivity test presented here is designed to establish the nature and scale of any measurable changes in blood cells that have been activated by the action of PMA on protein kinase C (PKC) and the subsequent activation of the superoxide generating NADPH oxidase system. Hence, if any of the cells were activated by the Ca-P bioceramic materials tested in this work, this would also show up as an elevated luminescent signal

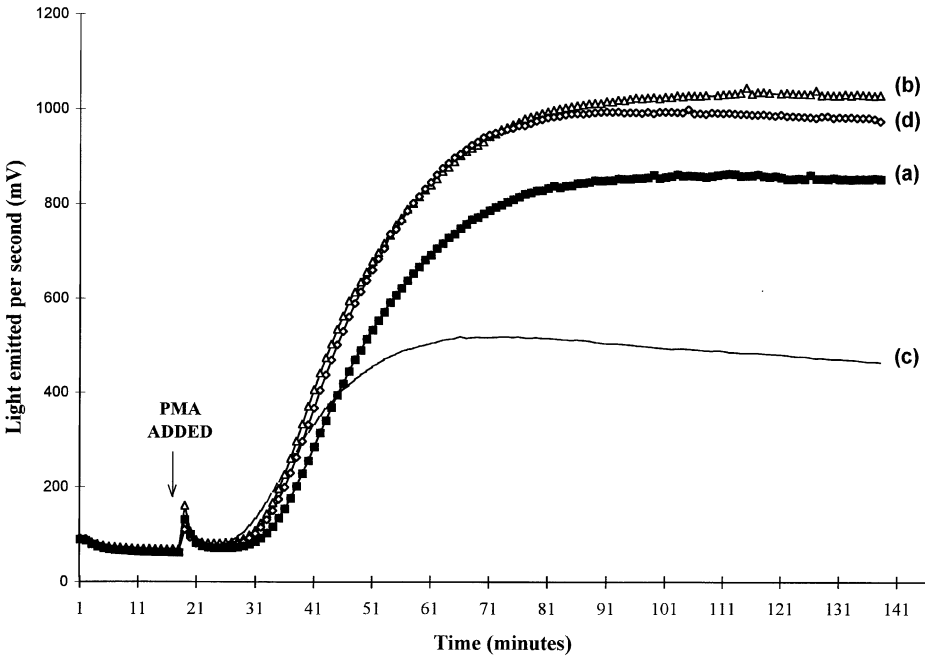

Fig. 1. Plots of light output (mV) versus time (min) for heparinised whole blood that has been exposed to (a) TCP for 34 min, (b) PBS control for 23 min. (c) TCP for 143 min. and (d) PBS control for 154 min.

prior to the addition of PMA and a relatively low luminescent response after stimulation.

Trace (a) in Fig. 1 shows the pre- and post-stimulation (PMA) light output recorded after exposure of a sample of heparinised whole blood to 1 mg ml^{-1} of TCP for a period of 34 min. Plot (b) shows the curve obtained after exposure of a control solution to the same blood sample for a similar time period. Scan (c) is the light output response after exposure of TCP sample for 143 min and plot (d) is the response of the corresponding PBS control solution after exposure for 154 min. Compared to the maximum light output for the PBS control, calculated as the average of traces (b) and (d), exposure to the TCP for 34 min resulted in a 15.7% reduction in peak chemiluminescence. Increasing the exposure time to 143 min produced a 49.3% reduction in peak chemiluminescence. By comparison, a reduction of only 4.1% was observed in the control (PBS) solution after the longer (143 min) incubation period.

This reduction in peak luminescence with increased exposure time to heparinised whole blood was a feature of all materials studied and was reproducible from blood sample to blood sample, irrespective of whether the sample was from volunteers who were known to suffer from rheumatoid arthritis or from normal controls. Clearly, relatively long exposure times were required to elicit a maximum response in whole blood samples. Hence, in an attempt to shorten the exposure time required to produce a suitable change in light output, various experiments were carried out using diluted whole blood samples.

3.2. Chemiluminescence of diluted blood after exposure to calcium phosphate powders

The use of heparinised whole blood diluted 1:100 with blood dilution buffer, but still containing the same concentration of powder as that used for the previous whole blood assay, was found to result in the same pattern of cell priming. However, the response maxima now occurred after a much shorter exposure time. Although the effect could be observed more-or-less immediately after the powder was added, the method was standardised so that each assay commenced precisely after a 6.5 min exposure to the powder samples.

Typical chemiluminescent responses of diluted blood after priming by exposure to (a) 1 mg ml^{-1} commercial TCP, (b) 1 mg ml^{-1} of commercial TCP thermally processed at 800°C (considered in detail later) and (c) the corresponding control, in which 100 μl PBS was substituted for the powder suspension are shown in Fig. 2. The individual responses to the five different blood donor samples (A–E) are given in Table 1. From these data it can be determined that exposure of the TCP to the diluted blood samples taken from each of the donors, gives a mean reduction in peak chemiluminescence of 39.4% (±2.7% cv) compared to that recorded for the PBS control (100%).

The light output versus elapsed time curves recorded after exposure of commercial grade HA material to diluted blood, pre- and post-stimulation with PMA, are shown in Fig. 3. As before, (a) shows the curve recorded for exposure to 1 mg ml^{-1} of HA, (b) exposure to

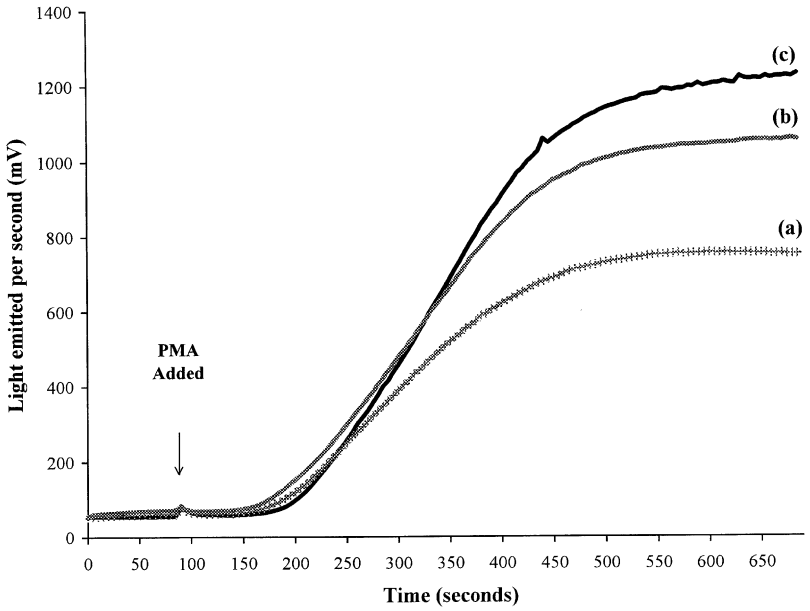

Fig. 2. Plots of light output (mV) versus time (s) for heparinised diluted blood (1:100) that has been exposed to (a) as received TCP, (b) TCP heated to 800°C and (c) the corresponding PBS control.

Table 1
Quantitative chemiluminescence data (light output in mV) for diluted blood (1:100) samples from five individual donors (A–E) after exposure to "as received" and thermally conditioned Ca-P powders

Material	Blood donors					Mean	Standard deviation
	A	B	C	D	E		
Thermally processed (800°C) HA(MW) Reduction in peak maximum (%)	15	28	14	23	30	22.0	6.0
Thermally processed (800°C) HA(MW) Light output (mV)	762	674	1030	769	805		
HA(MW) Reduction in peak maximum (%)	25	34	29	28	31	29.4	2.5
HA(MW) Light output (mV)	673	617	842	722	787		
Thermally processed (800°C) HA Reduction in peak maximum (%)	25	23	22	16	23	21.8	2.3
Thermally processed (800°C) HA Light output (mV)	671	724	930	844	882		
HA Reduction in peak maximum (%)	37	61	46	51	54	49.8	6.6
HA Light output (mV)	563	369	638	488	524		
Thermally processed (800°C) TCP Reduction in peak maximum (%)	13	10	11	9	11	10.8	1.0
Thermally processed (800°C) TCP Light output (mV)	781	845	1059	914	1022		
TCP Reduction in peak maximum (%)	36	43	36	41	41	39.4	2.7
TCP Light output (mV)	578	534	759	590	680		
PBS control Light output (mV)	898	940	1191	1002	1147		

$1\,mg\,ml^{-1}$ of a HA sample heated at 800°C for 2 h and (c) the corresponding (PBS) control. The individual responses to blood samples A–E are also reported in Table 1. The addition of this material to the five individual diluted blood samples was found to give an average reduction in peak height of 49.8% (±6.6% cv) compared to that recorded for the relevant control sample as given in Fig. 4 and Table 1.

The HA type material produced by the microwave assisted synthesis method was also assessed in the same manner and the responses are again shown in Table 1. In this case, the addition of $1\,mg\,ml^{-1}$ of this powder to blood samples from the five different donors gave an average reduction in peak height of 29.4% (±2.5% cv) compared to that for the PBS control solution.

3.3. Chemiluminescence of diluted blood exposed to heat-treated calcium phosphate powders

To examine the effect of thermal processing on the bioactivity of the various Ca-P systems of interest, samples of each material were thermally conditioned at 800°C, in air, prior to exposure to diluted blood. Measurement of chemiluminescence after stimulation with PMA carried out as before.

On exposure to diluted blood from the five individual donors, the thermally processed TCP produced an average reduction of 10.8% (±1.0% cv) in peak height compared to PBS (100%). The effect of thermal processing, therefore, was to reduce the much higher inhibition (39.4%) recorded for the as received TCP by

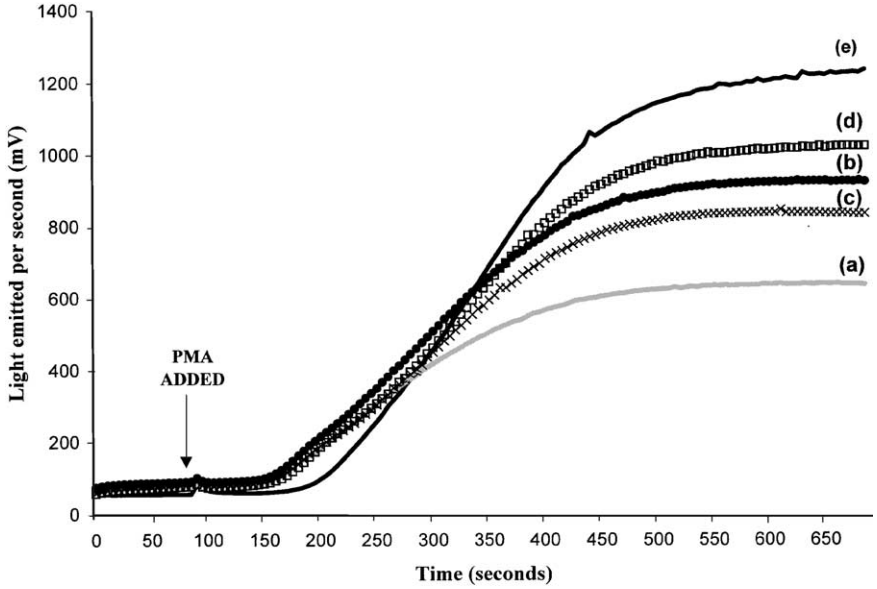

Fig. 3. Plots of light output (mV) versus time (s) for heparinised diluted blood (1:100) that has been exposed to (a) HA, (b) HA heated to 800°C (c) HA(MW) (d) HA(MW) heated to 800°C and (e) the corresponding PBS control.

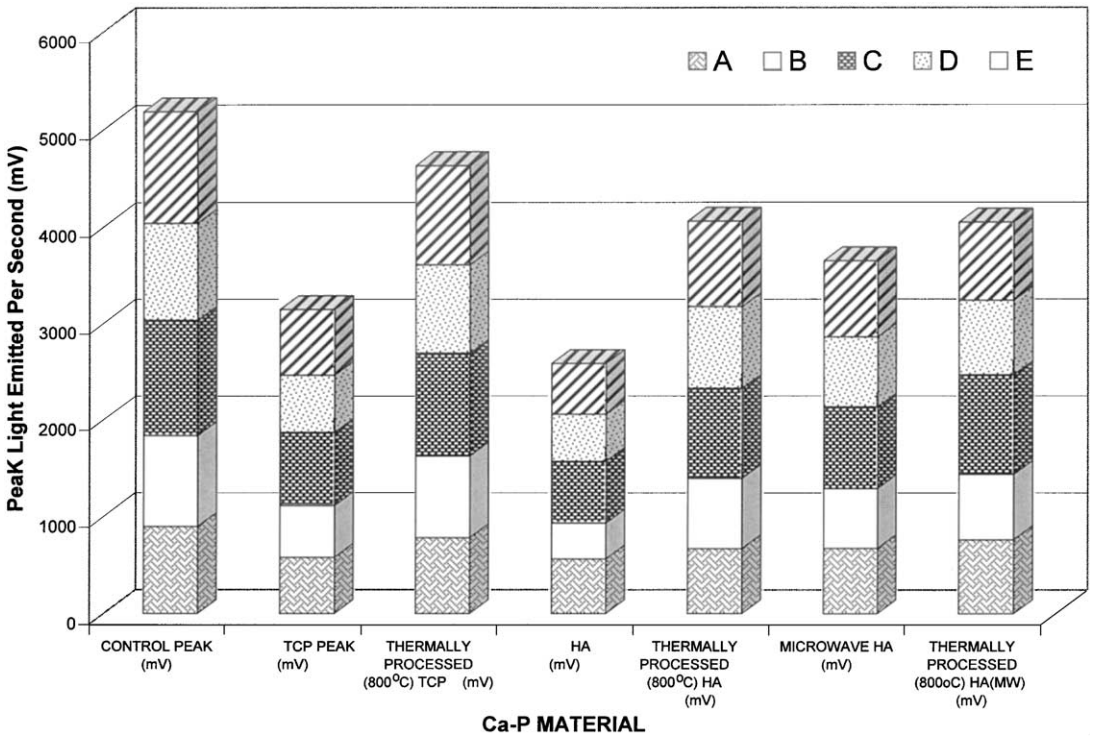

Fig. 4. Histogram of peak light emission per second (mV) for the six Ca-P materials (as received and heated 800°C) obtained after exposure to heparinised diluted blood (1:100) from each of the five blood donor samples.

28.6%. Thermally processed HA gave a 21.8% (±2.3% cv) inhibition in peak height, representing a reduction of 28% when compared to the as received equivalent (49.8%). The HA(MW) material led to an inhibition of light output of 29.4%. After heating to 800°C the inhibition was found to be 22% (±6% cv) as compared to its control (PBS) value.

Hence, for the as received systems, the commercial HA gave the greatest average reduction in peak chemiluminescence (49.8%), TCP gave the next

(39.4%) and HA(MW) gave the least change (29.4%). After thermal conditioning, the ranking order was found to reverse, with HA(MW) giving the greatest reduction (22%), closely followed by that for the commercial HA (21.8%) with TCP giving the least change (10.8%).

A direct comparison of the stimulated chemiluminescence results obtained from the exposure of the six different bioceramic powders (as received and thermally processed) to the blood from the five different donors is shown graphically in Fig. 4. From these effected cells it is clear that, whereas there was no significant difference between the way the individual samples of blood responded on exposure to each of the bioceramic materials, there were very big differences in the way the different powders the cells. Our ranking of bioactivity obtained from the results of this study is therefore: HA > TCP > HA(MW) > Thermally conditioned (800°C) HA(MW) > Thermally conditioned (800°C) HA > Thermally conditioned (800°C) TCP. These results suggest that, thermally conditioning the Ca-P powders reduces their bioactivity.

3.4. Chemical and structural characterisation of the calcium phosphate materials

An initial appraisal of these data would suggest that the ranking order of relative bioactivity for the Ca-P systems tested and reported here does not correlate directly with their perceived biofunctionality based on the assumed chemical and structural properties of each material. Hence, the differences noted between the predicted and measured responses led the authors to investigate the actual composition of the various powders before and after thermal processing, by FTIR spectroscopy and XRD.

The FTIR spectra for the as-received and thermally conditioned Ca-P materials are shown Fig. 5 (a)–(f). In all cases, absorption bands characteristic of P–O stretching vibrations (1097, 1036 and 963 cm^{-1}) and O–P–O bending vibrations (603 and 566 cm^{-1}) are clearly evident. The peaks at 3571 and 630 cm^{-1} are assigned to the hydroxyl O–H stretching and O–H libration bands, respectively. The broad band in the range 3700–3000 cm^{-1} can be attributed to traces of water with the band at 1640 cm^{-1} indicative of the associated H–O–H bending mode. Various amounts of a carbonate (CO_3^{2-}) species were also detected as indicated by the weak bands at 1415, 1458, 1543 cm^{-1}, an asymmetrical envelope centred on 879 cm^{-1} and a weak band at 1488 cm^{-1}. The weak bands around 2200–2000 cm^{-1} are associated with overtones and to combination bands of other spectral components.

The FTIR results for the as-received TCP (Fig. 5(a)) indicate sharp peaks for O–H stretching and librational bands more characteristic of HA than pure TCP. Indeed, this spectrum is almost indistinguishable from

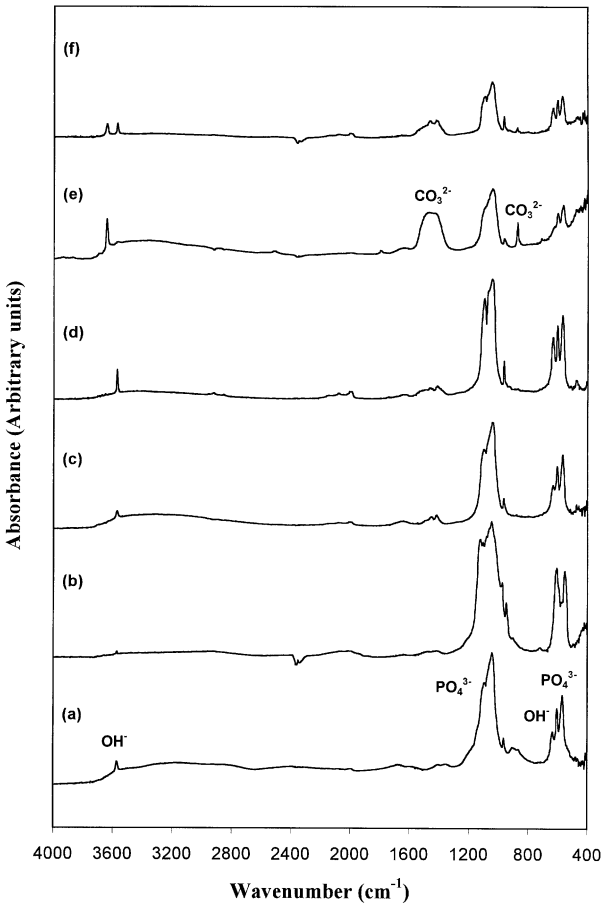

Fig. 5. FTIR spectra (4000–400 cm^{-1}) for (a) TCP, (b) TCP heated to 800°C, (c) HA, (d) HA heated to 800°C, (e) HA(MW) and (f) HA(MW) heated to 800°C.

that for the HA sample (Fig. 5(c)). After thermally processing this material to 800°C the FTIR scan shows significant changes. The O–H librational band (630 cm^{-1}) has disappeared and an O–H stretching vibration at 3571 cm^{-1} is barely visible. In addition, the PO_4^{3-} bands (1130–900 and 610–530 cm^{-1}) are indicative of both TCP and HA. The bands associated with CO_3^{2-} are also reduced in intensity. The broad band associated with water (3500–3000 cm^{-1}) has almost disappeared along with the H–O–H bending groups at 1640 cm^{-1}.

The FTIR data for the HA material (Fig. 5(c)) suggests CO_3^{2-} substitution at both the OH^- (A-site) and PO_4^{3-} (B-site) positions consistent with a previous analysis of this system as a carbonated apatite with relatively poor crystallinity [23]. The equivalent plot for this material after heating to 800°C (Fig. 5(d)) shows a marked increase in the intensity of the OH^- vibration. The bands associated with the P–O stretching and O–P–O bending vibrations also show improved resolution and assigned to CO_3^{2-} are still present. As expected, those spectral features associated water have decreased in intensity but do not completely disappear.

The FTIR spectrum for the as received HA type material synthesised using the microwave irradiation method (Fig. 5(e)) shows strong peaks associated with OH^- and CO_3^{2-}. However, the O–H librational band at 635 cm^{-1} appears only as a weak shoulder here. There is also evidence indicating that the CO_3^{2-} is substituting for PO_4^{3-} (B-site substitution) in the crystal lattice [24]. The weak band detected at 3643 cm^{-1} is ascribed here to the presence of $Ca(OH)_2$ [25]. On heating this material to 800°C several changes can be observed in the FTIR data (Fig. 5(f)). Bands in the region 1200–900 cm^{-1} (P–O stretching) and 700–400 cm^{-1} (O–P–O bending) all show enhanced resolution. Conversely, those features associated with CO_3^{2-} have decreased in intensity. Interestingly, two bands are now detected in the 3500–3700 cm^{-1} range and these have both been assigned to O–H stretching at 3571 and 3643 cm^{-1} associated with HA and ($Ca(OH)_2$), respectively.

The XRD patterns for the six (as received and thermally processed) Ca-P materials are given in Fig. 6(a)–(f). The X-ray diffractogram for the as-received TCP sample (Fig. 6(a)) shows clearly the presence of several Ca-P phases, none of which correspond directly to that for TCP when compared to the relevant Joint Committee on Powder Diffraction Studies (JCPDS) File 09-0169. The most prominent peaks are indicative of poorly crystallised HA with 2θ values at 25.8°, 31.8°, 32.2° and 32.9° corresponding to the 002, 211, 112 and 300 reflections, respectively (JCPDS File 09-0432). The other major phase present appears to be that of DCP (JCPDS File 09-0080) with 2θ values at 26.4°, 26.5°, 30.1° and 41.8°, corresponding to 020, 220, 112 and 013 reflections, respectively. On heating this sample to 800°C, the resulting diffraction pattern (Fig. 6(b)) and the subsequent 2θ values correspond closely to the data expected for crystalline TCP (JCPDS File 09-0169). The percentage crystallinity of the various Ca-P powders has also been determined from the XRD scans. For TCP an increase from 55% to 65% crystallinity is observed on heating the powder to 800°C.

For the as-received HA powder (Fig. 6(a) a series of broad peaks are observed at 2θ values that correspond closely to those indicated for HA (JCPDS File 09-0432). The four strongest peaks are assigned as 002, 211, 112 and 300 reflections corresponding to 2θ values of 25.8°, 31.8°, 32.2° and 32.9°, respectively. The poorly resolved nature of the peaks indicates a lack of crystallinity in this sample, but do signify that the material is indeed HA. After thermal processing at 800°C (Fig. 6(b)), all spectral lines are better resolved, indicating that at this temperature the material has enhanced crystallinity, as confirmed by the change in the measured percentage crystallinity which increases from 49% to 72% on thermal processing (Table 2).

The X-ray diffraction pattern obtained for the HA type material prepared by a microwave assisted synthesis (Fig. 6(e)) is indicative of HA (JCPDS File 09-0432). However, the peaks are quite broad suggesting that this material is quite amorphous. Furthermore, a peak that is characteristic of $Ca(OH)_2$ can be observed at a 2θ value of 18.0°. Additional $Ca(OH)_2$ peaks, especially the 100% intensity line expected at 34.1° 2θ may be masked by the relatively broad peaks for HA in the 2θ region 30–35°. After heating to 800°C, the resulting diffractogram (Fig. 6(f)) shows the presence of two distinct Ca-P phases namely, HA and TCP. Strong lines at 25.8°, 31.8°, 32.2° and 32.9° 2θ corresponding to the 002, 211, 112 and 300 HA reflections, respectively, while the peaks at 27.8°, 31.1° and 34.5° 2θ, correspond to the TCP 214, 0210 and 220 reflections. For this material, the increase in the percentage crystallinity after heating to 800°C is from 46% to 65% (Table 2).

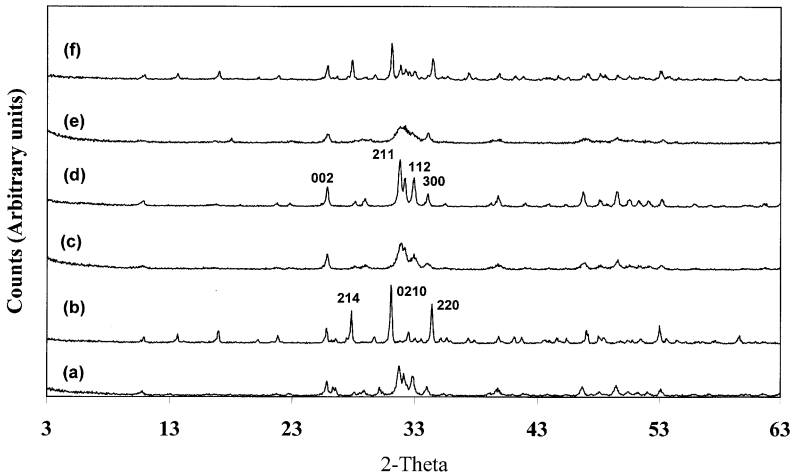

Fig. 6. XRD diffractograms recored in the range 3–63 2θ for (a) TCP, (b) TCP heated to 800°C, (c) HA, (d) HA heated to 800°C, (e) HA(MW) and (f) HA(MW) heated to 800°C.

These FTIR and XRD data clearly illustrate that the chemical and microstructural properties of the Ca-P powders used in these studies are significantly different than that expected from consideration of their assumed specification alone. In particular, it is apparent that the as received TCP and HA(MW) materials are not chemically pure. Heating the materials to 800°C clearly influences their biofunctionality. The attendant increase in percentage crystallinity for all three Ca-P systems makes them more chemically stable and therefore less likely to undergo dissolution and thereby interact with the leucocytes. The fact that the thermally processed TCP is determined to be the least bioactive is therefore due to the pronounced changes it undergoes on heating resulting in a stable mixed Ca-P material rather than the bio-resorbable system that it might otherwise have been expected to be. Hence, based on their actual chemical and microstructural properties, the ranking order of the Ca-P materials is indeed consistent with that determined by the chemiluminescence test described here, i.e. HA > TCP > HA(MW) > Thermally conditioned (800°C) HA(MW) > Thermally conditioned (800°C) HA > Thermally conditioned (800°C) TCP.

Table 2
Ca/P ratio of as received and thermally conditioned (800°C) Ca-P powders determined from EDX and XRF (as received only) analysis

Material	Ca/P	
	EDX data	XRF data
TCP	1.40±0.01	1.45±0.002
Thermally processed (800°C) TCP	1.39±0.02	
HA	1.67±0.02	1.67±0.002
Thermally processed (800°C) HA	1.63±0.01	
HA(MW)	1.58±0.02	1.69±0.002
Thermally processed (800°C) HA (MW)	1.58±0.03	

3.5. Possible quenching by calcium phosphate powders of light emitted from pholasin®

From the results summarised in Table 1, it is clear that the maximum response (light output) in the chemiluminescence tests was significantly reduced when the heparinised blood (diluted or undiluted) was exposed to various Ca-P materials. To assess whether this reduction in light output is as a consequence of a blood/material interaction or if it may possibly be due to free radical interactions with the Ca-P systems, or indeed direct inhibition of Pholasin® by the powders, two types of experiment were carried out. Firstly, the mean and peak light emitted from a sample of Pholasin® into which PMA was injected, was measured over 100 s, in the absence of blood, but in the presence and absence (control) of the powders. The responses observed for duplicate samples of TCP and HA are summarised in Fig. 7. These data then confirm that the presence of the bioceramic powders alone has no significant effect on the light output of the Pholasin®.

Secondly, superoxide was generated in the presence of Pholasin® and a control sample of PBS and the light emitted measured. This experiment was then repeated after addition of samples of the various Ca-P powders. If the materials were to compete with Pholasin® for superoxide then a reduction in luminescence would occur in the presence of each powder. The main plot in Fig. 8 gives an example of a typical reaction with serum where competition for superoxide between Pholasin® and serum led to a 44% reduction in light. The inset in the same figure presents typical data from three

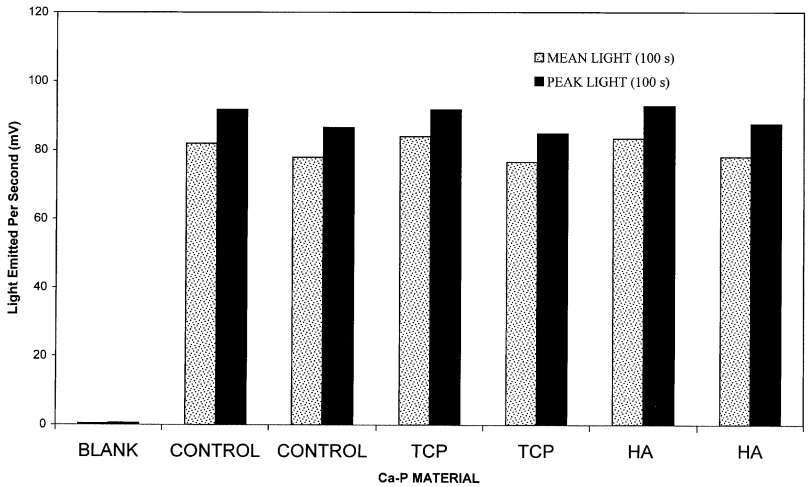

Fig. 7. Histogram of the mean and peak light output (mV) measured over 100 s from solutions containing Pholasin® and PMA only (no blood) in contact with as received TCP and HA. Data from the corresponding controls are also shown.

experimental runs in which a solution containing HA powder is used instead of serum. It can be seen that no significant reduction in light occurred in the present of the powder. These experiments were also repeated (in triplicate) with the TCP powder and no significant differences were found to occur. Hence, the results from both types of experiment show conclusively that there was no quenching of light or free radicals by the powders.

3.6. Cytotoxicity studies of calcium phosphate powders

To determine whether or not the observed reduction in peak chemiluminescence might be a consequence of cell death caused by contact with the Ca-P materials, the viability of polymorphonuclear leucocytes isolated from the samples exposed to each of the powders was determined by the trypan blue exclusion method. Tests were performed before and after the addition of the bioceramic powders to the blood and in all cases >99% cell viability was observed.

4. Discussion

PMA, the stimulant used in this chemiluminescent test, is a well-characterised soluble ligand that migrates into blood cells. PMA acts as a diacyl glycerol (DAG) analogue activating protein kinase C directly and leading to a fully functional assembled complex of proteins making up the NADPH oxidase system [26]. The activated NADPH oxidase is then responsible for the production and excretion of large amounts of the superoxide anion $O_2^{-\bullet}$.

Pholasin®, the 34 kD photoprotein from the bioluminescent marine mollusc, *Pholas dactylus* emits light in the presence of the free radical, superoxide. Hence, it is an ultrasensitive detector of any extracellular superoxide produced by activated neutrophils [18,19]. In addition, Pholasin® can also detect degranulation of myeloperoxidase in cells stimulated with PMA [20,27]. Here, PMA is used as a tool to stimulate blood cells to produce superoxide with Pholasin® acting as a light-emitting reporter of the response of the cells, before and after exposure to the bioceramic powders of interest.

The assay used here is designed to probe (a) whether exposure of the calcium phosphate powder to granulocytes in whole blood leads to activation of the cells and/or (b) if exposure of the powder to these granulocytes changes the way the cells will respond to stimuli presented after exposure to the powder. If any of the blood samples had cells which were activated by the powders, this would show up as an elevated luminescent signal prior to addition of PMA and a relatively low luminescent response after stimulation with PMA.

The fully active NADPH oxidase is a membrane-bound complex consisting of a short electron transport chain [27] (flavocytochrome b_{245}) and four cytosolic

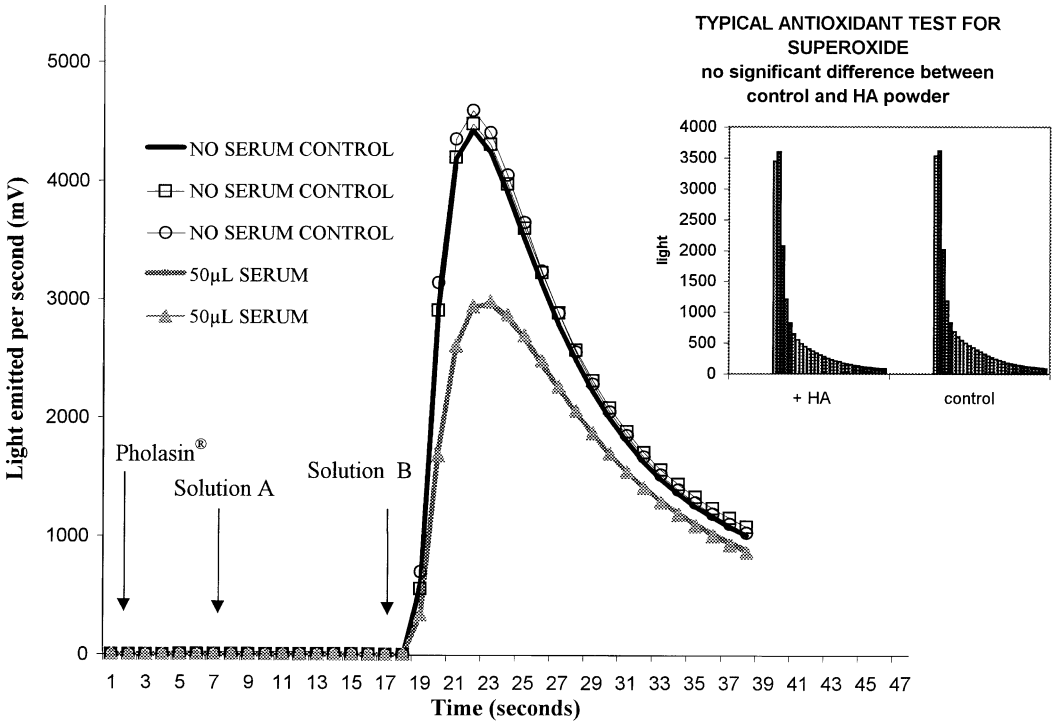

Fig. 8. Plots of light output (mV) versus time (s) illustrating a 44% reduction in luminescence caused by competition for superoxide by addition of serum. Insert shows no significant reduction in light output caused by exposure to HA compared to that from the corresponding PBS control.

proteins: $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and $p21^{rac}$; where the superscript suffix "phox" represents phagocyte oxidase. The induction of electron transport is most likely related to translocation of cytosolic proteins to the membrane as that event coincides with oxidase activity [28]. The $p21^{rac}$ is a GTP binding protein, which occurs in the cytosol as a complex with Rho-GDI, a GDP-dissociation inhibition factor. The function of the oxidase is to accept an electron from NADPH and transfer it to the inside of the cell membrane from where it is passed to oxygen on the outside wall. This one electron reduction of oxygen leads to the direct production of superoxide at the surface of the cell. The dissociation of Rho-GDI from $p21^{rac}$ is the most probable switch that initiates the activation of the oxidase [26]. Upon activation, $p21^{rac}$ dissociates from Rho-GDI and translocates to the cell membrane [29] where an activation complex involving $p21^{rac}$, $p47^{phox}$ and $p67^{phox}$ couples with flavocytochrome b_{245} inducing a conformational change conducive to electron transport [27]. NADPH, the substrate and the source of the electrons for reduction of oxygen, is produced from the oxidation of glucose via the hexose monophosphate shunt. This so-called respiratory burst of phagocytes has been recognised for some time as being distinct from mitochondrial respiration [30].

Whereas, there are still some gaps in our understanding of the mechanism of the NADPH oxidase system, a considerable amount of information on the structure, function and mechanism of activation is known. It is now accepted [31] that the oxidase remains inactive until exposed to appropriate stimuli [27]. Stimulation can occur in two ways, namely: via ligands which bind to receptors such as the complement fragment C5a, *N*-formylated methionylpeptide (fMLP) derived from bacterial cell walls, bioactive lipids such as platelet activtating factor (PAF) and leukotriene B4 (LTB4) or by direct agonists of protein kinase C (PKC) such as PMA or diacyl glycerol. These two mechanisms for activation of the NADPH oxidase in neutrophils are distinct and Pholasin® can quantify both types [20,32]. The receptor ligand fMLP is known to identify neutrophils which have been activated by exposure to haemodialysis filters and oxygenators used during cardiopulmonary by-pass surgery as well as bacterial endotoxin [32–35]. Since in this study, two of the blood donors (A and E) were receiving treatment for rheumatoid arthritis, PMA was deliberately chosen as the sole stimulant because, unlike the response to stimulation with fMLP, the kinetics of the response to PMA appear to be independent of disease state. Hence, it is primarily the action of PMA on protein kinase C (PKC) and the subsequent activation of the superoxide generating NADPH oxidase system in the presence of the calcium phosphate powders that is being addressed.

The explanation for this phenomenon can be explained by three possible hypotheses, namely: (I) that the effect is due to reduced activity of the NADPH oxidase perhaps by an affect on PKC; (II) there is an increased consumption of superoxide by intracellular myeloperoxidase or (III) phagocytosis of the particles results in the utilisation of superoxide by myeloperoxidase during the production of hypochlorous acid. Whereas, hypothesis (I) has not been tested here, hypothesis (II) is consistent with previous findings in which enhanced chemiluminescence (with Pholasin®) occurred in neutrophils, stimulated with PMA, which were deficient in myeloperoxidase which reacts rapidly with superoxide produced by the NADPH oxidase [34]. Further support for hypothesis (II) comes from the observation that 5×10^5 neutrophils, primed by exposure to hydroxyapatite demonstrated enhanced chemiluminescence in the presence of luminol which reports mainly the myeloperoxdase activity within the cell. While neutrophils are capable of phagocytosing particles, hypothesis (III) can be eliminated on the grounds that the effect of carrying out the test with 1:100 diluted blood reduced the time needed for incubation of the powder with the blood in order to obtain the maximum response. As described earlier, the time delay between exposure of the blood to the powder and injection of the PMA was standardised to 6.5 min which was the minimum time needed to set up the assay, equilibrate the reagents and inject the stimulant. Preliminary trials, however, revealed the effect to occur in much less time. As phagocytosis by neutrophils usually takes about 20 min to commence our results cannot, therefore, be attributed to this phenomenon.

5. Conclusions

In this study, the stimulatory effects on human leucocytes caused by a series of Ca-P bioceramic materials has been assessed using a chemiluminescence method based on the photoprotein Pholasin®. All the materials studied caused significant levels of inhibition to the expected light output compared to relevant controls. No differences were found in the relative responses of the powders in undiluted or diluted (1:100) blood assays. However, a large improvement in the speed of response occurred when the experiments were carried out in the diluted blood.

Comparison of the degree of inhibition of stimulated luminescence resulting from exposure of diluted blood to the various bioceramic powders can be summarised as follows: HA > TCP > HA(MW) > Thermally conditioned HA(MW) > Thermally conditioned HA > Thermally conditioned TCP. An initial consideration of this ranking order as a scale of bioactivity did not correlate well with the assumed chemical and structural properties

of each of the Ca-P materials. However, a detailed determination of the chemical and structural composition of the materials of interest, before and after thermal processing, by Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) indicated that the actual properties of as received TCP and HA(MW) were different than expected. Therefore, the results reported using this new chemiluminescence assay with Pholasin® apparently gave more information concerning the relationship between the composition of the material and the response it elicits in leucocytes in blood exposed to it. Indeed, it is reasonable to suggest that the test protocol indicated the true nature of the Ca-P powders prior to the analytical studies.

The new method reported here clearly has the potential to become a very useful test for assessing the bioactivity of Ca-P and related materials. The speed of response observed for the optimised experimental protocol suggests that it has the capability of performing high throughput screening. Thus, coupling the diluted blood chemiluminescence technique with a multi-sampling device has real potential to provide for high throughout screening of bioactive materials. Development of the technique and its further application to the screening of other types of biomaterials is currently underway.

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