

IN VITRO TESTING OF CALCIUM PHOSPHATE WHISKERS FORMED BY A MICROWAVE-ASSISTED MOLTEN SALT PROCESS

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ABSTRACT

Calcium phosphate (single-phase HA, single-phase TCP, and biphasic HA-TCP) nanowhiskers and/or powders were produced by using a microwave-assisted "combustion synthesis (auto ignition) /molten salt synthesis" hybrid route.

Aqueous solutions containing NaNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and KH_2PO_4 (with or without urea) were irradiated in a household MW oven for 5 minutes at 600 watts of power. The as-synthesized precursors were then simply stirred in water at RT for 1 hour to obtain the nanowhiskers or powders of the desired calcium phosphate (CaP) bioceramics. *In vitro* testing (cytotoxicity, cell viability, and total protein assay) of the whiskers was performed by using the mouse osteoblast cells (7F2). These bioactive and osteoconductive whiskers/fillers are designed for use in biopolymer-based bone substitute scaffolds.

INTRODUCTION

Calcium hydroxyapatite (HA: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the major inorganic phase of bones, is a member of the "apatite" family. However, biological apatites (e.g., enamel, dentin, and bone), differ from pure and synthetically produced calcium hydroxyapatite in stoichiometry, composition, crystallinity, and also in other physical and mechanical properties [1].

Minor cations (e.g., Na^+ , K^+ , Mg^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} , Zn^{2+} , or Fe^{2+}), and anions (e.g., HCO_3^- , HPO_4^{2-} , Cl^- , and F^-) are also associated with biological apatites.

These can be considered as ionic substituents in the apatite structure. The more resorbable β -TCP phase (tricalcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$), when present in synthetic bone substitute materials, helps improving the resorption and bioactivity of the implanted bioceramics, and increase their ability in taking part in the *in vivo* bone remodeling processes.

HA or TCP powders have generally been synthesized from aqueous solutions for use in bioceramic applications. It is known [2] that calcium hydroxyapatite is the least soluble and the most stable calcium phosphate phase in aqueous solutions at pH values higher than 4.2. HA has been synthesized either in neutral or highly alkaline media [3-6] to ensure the thermal stability of the formed phase after high-temperature (1100°-1300°C) sintering.

Synthesis of HA in neutral or slightly acidic media [7] is known to be a more complicated and difficult task. The synthesis of biphasic mixtures of HA and TCP has also been achieved by aqueous coprecipitation [8]. HA-TCP biphasic bioceramics, owing to the higher *in vivo* solubility of β -TCP, display increased bioactivity as compared to more stable pure HA samples [9].

This paper utilizes a microwave-assisted materials [10] combustion synthesis (CS) b purity α -alumina powders. W from "Combustion Synthesis" mostly solid reactants [13. 1 fuel in a $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and ously used to synthesize calci acid and ethylene glycol. mi Pechini process [16] was also phate powders [17]. However and Pechini-like process [17] 1200°C for prolonged period (i.e., HA, TCP or biphasic). 1 synthesis (MSS). Molten salt shown to form monodisperse ture of HA powders and K_2SO_4 to remove the K_2SO_4 glass a munication reports the devel assisted, molten salt-combu manufacture nanowhiskers a bioceramics. The work is an utilizes the useful features of

EXPERIMENTAL PROCEDURE

Aqueous solutions containing HNO₃ and urea (all re prepared in 10 mL of deionized Table I. The first chemical a one was urea. Small amount purpose of dissolving back a following the addition of Ca- each case shown in Table I. beakers were then placed on covered with an upside down auto ignition-molten salt proc into a household microwave Boca Raton, FL) for only 5

Sample	NaNO_3 (g)	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (g)
HA	5.00	1.00
TCP	5.00	1.00
Biphasic	5.00	1.00

Table I
Sample Preparation for the

This paper utilizes a microwave-assisted "Auto Ignition" process to synthesize the aforementioned materials [10, 11]. The auto ignition synthesis has been referred to as combustion synthesis (CS) by Kingsley and Patil [12] for the preparation of high-purity α -alumina powders. We prefer to use the term auto ignition to distinguish it from "Combustion Synthesis" or "Self-Propagating High Temperature Synthesis" using mostly solid reactants [13, 14]. The process, which used urea (H_2NCONH_2) as the fuel in a $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{HPO}_4$ system of starting chemicals, was previously used to synthesize calcium phosphate powders [15]. A variant procedure (citric acid and ethylene glycol, mixed with $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $\text{NH}_4\text{H}_2\text{PO}_4$) based on the Pechini process [16] was also shown to work well for the synthesis of calcium phosphate powders [17]. However, both the combustion synthesis (or auto ignition) [15] and Pechini-like process [17] required high-temperature calcination steps (at 1000° to 1200°C for prolonged periods) for the formation of the calcium phosphate phases (i.e., HA, TCP or biphasic). This is where the advantage can be taken of molten salt synthesis (MSS). Molten salt synthesis technique [18], which uses a molten flux, was shown to form monodispersed whiskers of HA upon heating and cooling of a mixture of HA powders and K_2SO_4 from 1100° to 1200°C , followed by washing in water to remove the K_2SO_4 glass and expose the HA whiskers [19, 20]. The present communication reports the development of an extremely simple and rapid microwave-assisted, molten salt-combustion (auto ignition) synthesis hybrid procedure to manufacture nanowhiskers and nanopowders of HA, TCP and HA-TCP biphasic bioceramics. The work is an example of our synergistic processing technique that utilizes the useful features of the aforementioned processes.

EXPERIMENTAL PROCEDURE

Aqueous solutions containing dissolved NaNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, KH_2PO_4 , concentrated HNO_3 , and urea (all reagent-grade, Fisher Chemicals, Fairlawn, NJ) were prepared in 10 mL of deionized water in 30 mL-capacity Pyrex[®] beakers, as shown in Table I. The first chemical added and dissolved in water was NaNO_3 , while the last one was urea. Small amounts of concentrated nitric acid were added for the sole purpose of dissolving back any colloidal calcium phosphates that might have formed following the addition of Ca- and phosphate-salts into the NaNO_3 solution. Thus, for each case shown in Table I, clear-transparent solutions were obtained. Small sample beakers were then placed onto $10 \times 10 \times 1$ cm alumina insulating fiberboards and covered with an upside down 250 mL-capacity glass beaker. To proceed with the auto ignition-molten salt process, the above-mentioned sample assemblies were placed into a household microwave (MW) oven (Sunbeam, max. power 600 W, 2.45 GHz, Boca Raton, FL) for only 5 minutes.

Sample	NaNO_3 (g)	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (g)	KH_2PO_4 (g)	15.69 M HNO_3 (mL)	Urea (g)	Ca/P
HA	5.00	1.00	0.345	0.10	1.75	1.67
TCP	5.00	1.00	0.384	0.10	—	1.50
Biphasic	5.00	1.00	0.345	0.10	0.075	1.67

Table I
Sample Preparation for the HA, TCP, and HA-TCP Biphasic Whisker Samples

MW oven was always operated at its maximum power setting. At the end of 5 minutes of MW heating within a well-ventilated fume hood, the sample beakers were taken out and their contents were let to cool down to RT in ambient atmosphere over the next 10 to 15 minutes. The substance in the sample beaker was then scraped off with a spatula, and directly placed (without grinding) into 500 mL of deionized water at room temperature ($22 \pm 1^\circ \text{C}$) and vigorously stirred for 1 hour. Formed suspension was filtered by using a vacuum-assisted Buechner funnel, and washed with 2 L of water, prior to drying the powders at 80°C for 2 hours. Samples were characterized by powder XRD (XDS 2000, Scintag, Sunnyvale, CA), FTIR (Nicolet 550, Thermo-Nicolet, Woburn, MA), SEM (S-3500 and S-4700, Hitachi, Tokyo, Japan), FESEM (Hitachi S-4700), and ICP-AES (61E, Thermo Jarrell Ash, Woburn, MA) analysis. Mouse osteoblast cells (7F2, ATCC, Rockville, MD) were used for cell culture and attachment studies. Cells were first grown at 37°C and 5% CO_2 in alpha MEM, augmented by 10% FBS. The culture medium was changed every other day until the cells reached a confluence of 90-95%. Osteoblasts were seeded at a density of 10^5 cells/cm². Cell cytotoxicity measurements were carried out after 24 hours, cell viability assessment was performed after 72 hours and total protein amount were measured after 7 days. Adhesion of the cells was quantified 24 hours after seeding. Trypan blue was added and the cells were counted using an Olympus BX60 light microscope. Only cells that stain blue were deemed necrotic because of plasma membrane damage. For statistics, all experiments were performed in triplicate where $n=3$. Analysis of variance was performed using the Tukey-Kramer multiple comparisons test. Osteoblast morphology after attachment was further examined by using SEM, after sputter-coating the samples with platinum.

RESULTS AND DISCUSSION

Sample solutions placed into the MW oven first went through an initial stage (1 minute) of evaporation and boiling of water. Urea is known [21] to decompose into biuret ($\text{H}_2\text{N}-\text{CO}-\text{NH}-\text{CO}-\text{NH}_2$, i.e. $\text{C}_2\text{H}_5\text{N}_3\text{O}_2$), cyanuric acid (HCNO), and ammonia (NH_3), when it is heated to about 200°C . Biuret itself then decomposes when heated at temperatures $>300^\circ \text{C}$. Nitrate solutions, on the other hand, usually decompose at low temperatures with the evolution of gases, such as NO_2 , NO and N_2O_5 . Moreover, we used NaNO_3 , which melts at 307°C [22] at a high concentration in our liquid samples. It would have been possible to lower this salt melting temperature further if we were using mixtures of NaNO_3 and KNO_3 [23-29]. All the samples glowed red in the MW oven between the 90 and 240 seconds of heating time. In high urea solutions, intermittent sparks were also observed within that time period. This is the evidence of occurrence of combustion in this hybrid process. In the solutions containing urea, an exothermic reaction occurs which generates high temperatures leading to deformation of the Pyrex[®] beakers. At the end of 5 minutes of MW irradiation, the recovered sample beakers only contained a liquid phase of slightly yellowish tint. NaNO_3 did not evaporate and it formed a molten salt. This liquid phase solidified at RT within the following 4-5 minutes, by forming visible dendritic crystals of NaNO_3 on its surface. Powder XRD data of all the unwashed samples (i.e., after 5 minutes of microwave heating, but without wash in water) only showed the presence of NaNO_3 as the crystalline phase, as seen in Figure 1(a). However, as expected, FTIR data of the unwashed precursors revealed the presence of characteristic phosphate IR bands (labeled in Figure 1(b), as given in detail elsewhere [15, 19].

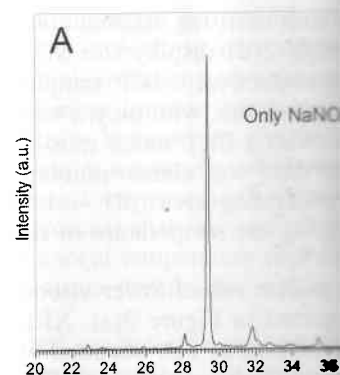


Fig. 1
(a) XRD and

Unwashed precursors were obtained (20 to 700 nm). Following dissolution and removal of the solution, the particles were raised to about 11.5-11.8 at the surface of the solutions at $t=0$ was

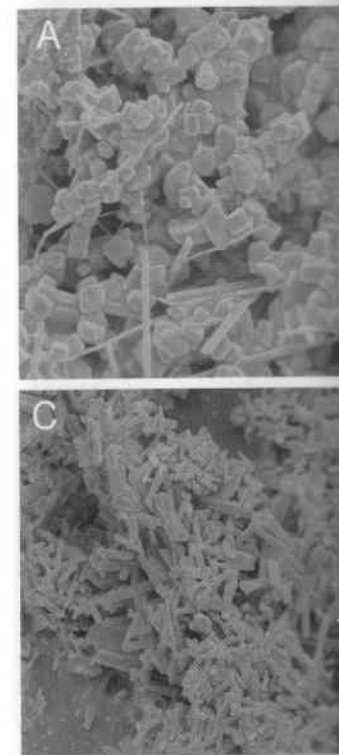


Fig. 2 SEM micrographs of

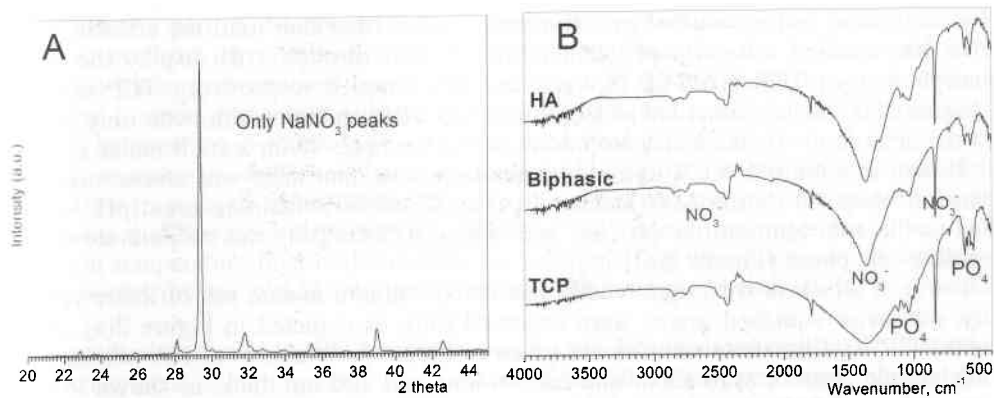


Fig. 1

(a) XRD and

(b) FTIR data (transmittance) of MW-heated, unwashed CaP precursors

Unwashed precursors were collected from the reaction beakers as relatively big chunks (20 to 700 mm). Following washing in water, these chunks turned porous, upon the dissolution and removal of molten NaNO₃. pH values of the washing solutions in situ raised to about 11.5-11.8 at the end of 1 hour (t=60 min) of stirring at RT. pH value of the solutions at t=0 was around 10.

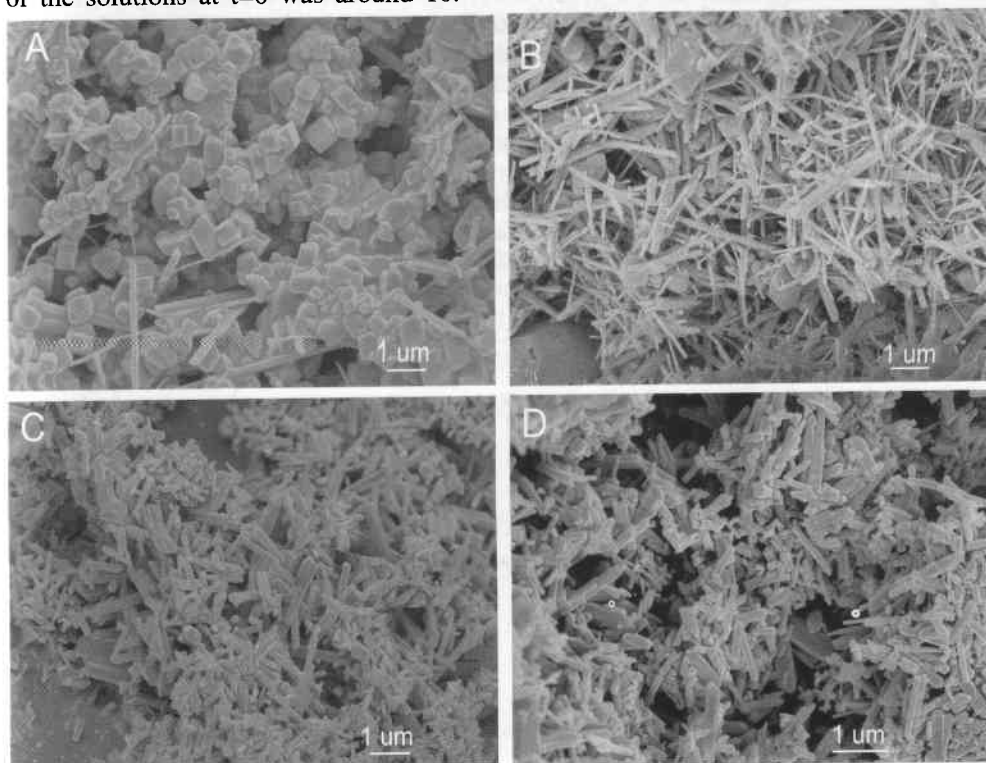


Fig. 2 SEM micrographs of (a) TCP, (b) HA-TCP biphasic, and (c) HA samples

Powders were further washed in a Buechner funnel filter unit until the effluent solution has reached a neutral pH value. Figures 2(a) through 2(d) display the SEM morphology of TCP, HA-TCP biphasic and HA samples, respectively. TCP samples (Figure 2(a)) mainly consisted of Whitlockite (β -TCP) rhombohedra, with only a very small amount of whiskers. The solutions of TCP samples (with a Ca/P molar ratio of 1.50 and solution pH \approx 0.9) did not contain any urea, and there was almost no combustion observed during MW irradiation. The solutions containing urea (pH \approx 1.5) led to the auto ignition-like process, and helped in increasing the temperature of the molten salt phase (Figure 2(b)).

Growth of whiskers was more readily facilitated within a molten salt of lower viscosity, otherwise equiaxed grains were observed [30], as depicted in Figure 2(a). XRD and FTIR data for these samples are given in Figure 3. Whiskers of HA obtained by this simple process were about one micron-long and 100 nm-thick, as shown in Figures 2(c) and 2(d).

The biphasic samples, Figure 2(b), contained around 60% TCP and 40% HA.

The volume percentage of the HA phase can easily be adjusted by varying the urea content in the starting solutions, as shown in Figure 4. ICP analyses of the HA, TCP and HA-TCP biphasic samples revealed that they contain around 1.5 wt% Na and 250 ppm K.

In the case of HA samples, such ionic substitutions surely causes a deviation from the perfect stoichiometry of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.

However, non-stoichiometry in an apatitic calcium phosphate sample is perfectly acceptable if the aim is to produce a synthetic biomaterial which can resorb *in vivo*, and readily take part in the bone-remodeling process [31]. Human bones contain a total of 1.3 wt% of cations like Na^+ , K^+ and Mg^{2+} [1].

The process developed here yields bone-like calcium phosphate samples, as confirmed by the FTIR data of Figure 3(b).

Carbonate ion concentration in human bones amounts to a significant 5.8 wt% of the total weight [1]. It is also a well-known fact that either synthetic hydroxyapatites [32] or human bones [33] heated above 700°C readily lose their carbonate ions.

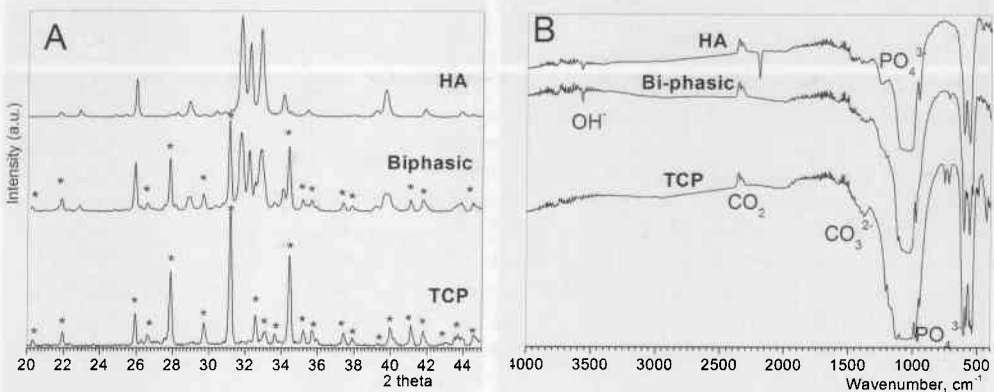


Fig. 3 (a) XRD (*: TCP peaks), and (b) FTIR data (transmittance) of washed CaP samples

An important part of the process were to perform the combustion (samples) under conventional chamber furnace, temperature. This is because the reaction through a container wall, coming from their precursors in solution. It is important that the particle growth, the local temperature distribution, and the temperature distribution would be

MW irradiation should lead to a solution system, achieving a uniform temperature during the preparation of submicron-sized particles. This process by the molten salt phase precipitates. Such precipitation is due to the excess water. Due to the observation (evidenced by the observations), and these *in situ* processes in a bath. Embryonic particles of nucleation and growth processes occur, its melting point down to room temperature. The preferred growth axes of the particles probably occurs during this "whisker-growth" process [33].

Solubility of the specific calcium phosphate (of temperature) has a significant effect. The degree of superheating of the solution, the resultant aspect ratio of the particles, and the growing out of the cooling rate, and the time at the peak temperature, and the follow-up studies, which were

Cell viability and protein adsorption histograms obtained with the tests using mouse osteoblasts (Figures 4 and 5, respectively). It is not abnormal to see in the tests performed with osteoblasts on more basic and nano-textured surfaces would allow an increase in the number of cells attached on them. Therefore, nanowhiskers of hydroxyapatite were able to register the high cell viability (Figure 4), in comparison to rhombohedra of TCP (Figure 2(a)).

An important part of the present work is the assistance of microwave heating. If we were to perform the combustion of our initial solutions (for the biphasic and HA samples) under conventional heating, for instance, in an electrical-resistance heated chamber furnace, temperature distribution of the solution would be inhomogeneous. This is because the reaction beakers would have been heated by thermal conduction through a container wall, convection and agitation. Since the synthesis of nanoparticles from their precursors in solutions consist of nucleation and particle growth, it is important that the particle growth is terminated [34]. Those processes partly rely on the local temperature distribution within the solution. Any inhomogeneity in the temperature distribution would then cause broad distributions in particle size.

MW irradiation should lead to homogeneous, i.e., molecular-level heating of the polar solution system, achieving automatic control of the above two processes required for the preparation of submicron-size whiskers or particles. The liquid phase formed during this process by the molten NaNO_3 dissolves all of the initially formed calcium phosphate precipitates. Such precipitates form during the initial boiling and evaporation of the excess water. Due to the presence of urea, an auto ignition process is initiated (as evidenced by the observation of intermittent sparks in the irradiated reaction beakers), and these *in situ* processes raise the extent of superheating in the molten-salt bath. Embryonic particles of calcium phosphate phases are formed through the nucleation and growth processes. As the molten ionic bath cools, while passing through its melting point down to room temperature, rapid crystallization occurs along the preferred growth axes of the ceramic phase. Calcium phosphate nanowhisker growth probably occurs during this rapid process, by a sequential "dissolution-crystallization-whisker-growth" process [35].

Solubility of the specific calcium phosphates in molten NaNO_3 (which is a function of temperature) has a significant role in the crystallization process to follow. The degree of superheating of the molten NaNO_3 must also have a strong effect on the resultant aspect ratio of the formed whiskers. The morphology of the whiskers growing out of the cooling molten salt bath could be strongly dependent on soaking time at the peak temperature, as well as the cooling rate. Such issues further warrant follow-up studies, which we plan to report in due time.

Cell viability and protein concentration histograms obtained with the cell culture tests using mouse osteoblasts are given in Figures 4 and 5, respectively.

It is not abnormal to see in cell culture tests performed with osteoblasts that the more basic and nano-textured substrate surfaces would allow an increased number of cells attached on them.

Therefore, nanowhiskers of hydroxyapatite were able to register the highest scores of cell viability (Figure 4), in direct comparison to rhombohedra of TCP as shown in Figure 2(a).

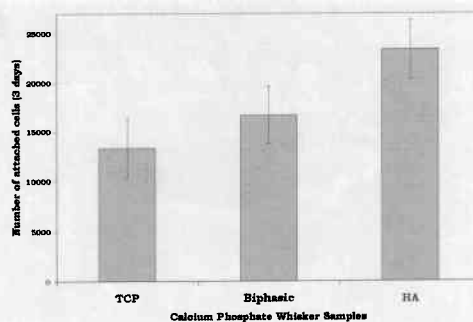


Fig. 4 - Cell viability