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(54) Title: METHOD OF MAKING MONETITE

(57) Abstract: The present invention relates to a method of making monetite comprising: mixing a solid phase comprising a basic calcium salt and optionally an acidic calcium salt with an aqueous phase, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and forming monetite. The invention also relates to compositions, cements and kits for forming monetite and the use of the compositions and cements.

# METHOD OF MAKING MONETITE

## Field of the Invention

The invention relates to a method of making monetite. Monetite can be used in dentistry, for example, as a bone substitute or as a support or scaffold for bone tissue engineering. The invention also relates to a composition, a cement and a kit which can be used to make monetite. Further, the invention relates to the use of the composition and the cement in surgery or therapy, and a method of treatment which makes use of the method of the invention.

## Background to the Invention

Significant bone defects arising from, for example, mal- or non-union of fractures, tumour resection, or post surgical trauma require bone grafting. It is estimated that about 2.2 million (Stok *et al.* (2010)) grafting procedures are carried out each year worldwide.

Autologous bone grafting is currently considered as the gold standard to restore bone defects. It is generally harvested from the iliac crest and this living bone contains the osteogenic factors that can stimulate bone formation; however, clinical benefits can vary as the cellular components may be damaged during transplantation and harvesting is also associated with donor site morbidity (Banwart (1995); Younger (1989)). Further, complications can occur and it is estimated about 8–39% show varying degrees of problems.

Synthetic bone substitutes are being extensively researched to produce a material that is osteoconductive, osteoinductive, has appropriate mechanical properties and has the ability to function as a delivery system for biologics. Ideally, the bone substitute should have mechanical properties similar to the bone being replaced and should allow cellular ingrowth, support new bone formation and degrade over time so that new bone can replace it. These are challenging requirements and recent studies have shown (Stok *et al.* (2010); Seebach *et al.* (2010)) that despite numerous bone substitutes being available the overall level of clinical evidence is low and there is a wide variation in the way a substitute supports cell survival and function.

Calcium phosphates have remained the material of choice for developing bone substitutes and or scaffolds for bone tissue engineering (De Groot (1983), Driskells (1973)). Calcium phosphates can be essentially divided into two categories: cements and ceramics. Hydroxyapatite (HA) derived grafts are ceramic in nature and are usually obtained through sintering at temperatures greater than 1000°C. α and β-tricalcium phosphate (α,β-TCP), sintered hydroxyapatite (SHA) and tetracalcium phosphate are other phases that can be obtained via sintering methods. Examples of such grafts are Cerabone®, Endobon®, ChronOS® (β-tricalcium phosphate), Bone Save® (composite of HA and TCP) and Actifuse ABX® (silicate substituted porous HA). In 1983, Brown and Chow (Brown (1983)) demonstrated that by

mixing one or several calcium phosphate powders with a liquid phase, a cement paste could be obtained which had the ability to harden at both room and physiological temperatures. The discovery of these new calcium phosphate cements (CPCs) allowed the possibility of treating bone defects by using mouldable pastes that were able to easily fill any defect shape. However, these cements are dense in nature, resorb slowly, are mechanically weak and require additives to promote fracture healing. Some examples of CPC cements in clinical use are Norian SRS (Synthes), Hydroset (Stryker), ChronOS™Inject (Synthes), BoneSource® (Stryker) and Calcibon® (Biomet).

The role of synthetic bone graft substitutes is currently changing and the aim is for the substitute to function as a scaffold for bone tissue engineering. Thus, it is not only important for the scaffold to integrate with the bone but exploit the potential for use as a cell seeded construct that can start the osteogenesis process.

**Calcium phosphate cements**

Calcium phosphate cements are obtained by mixing various calcium phosphate precursor phases with water or aqueous solutions as a result of precipitation of another phase. Depending on the pH of the chemical reaction, two types of cement are generally obtained: for pH $\geq$ 4.2, the reaction product is hydroxyapatite (HA) (Chow (1991)), and for pH $\leq$ 4.2, the product is dicalcium phosphate dihydrate (DCPD), also known as brushite (Mirtchie (1989)). Hydroxyapatite based materials have a low resorption rate due to its poor solubility at physiological pH. In contrast, brushite cements have raised considerable interest in the last decade because they are metastable under physiological conditions and can be resorbed more quickly than HA cements that are stable (Vereecke (1990)). Another calcium phosphate phase that shows a similar thermodynamic behaviour to brushite is dicalcium phosphate anhydrous (DCPA), also known as monetite.

The hardening process of CPCs is determined by two mechanisms which occur in supersaturated solution via nucleation and crystal growth. The latter is composed of several processes, starting with transport of ions from the bulk solution to the nuclei surface, followed by adsorption on energetically favourable sites. As mentioned above, the main products of this process are generally hydroxyapatite (HA) or dicalcium phosphate dihydrate (DCPD), commonly referred to as 'brushite'. However, there are a wide range of calcium phosphate phases that may precipitate, most likely as precursors to the most stable chemical phase that is expected to precipitate during the hardening process of the cement. Hence cements formed at a pH lower than about 4.2 need to be characterized because different phases can precipitate due to various kinetic factors. The most thermodynamically stable phase may not necessarily be the one that precipitates first because its precipitation kinetics may be slower.

Recently, many researchers have investigated the biological properties of monetite as a bone substitute scaffold. Preformed monetite can be obtained by modification of α or β-TCP blocks immersed in phosphoric acid solution (Laetitia (2008)) or by hydrothermal modification of brushite matrices that are

obtained from calcium phosphate cement formulations. One of the main reasons for exploring the monetite phase is due to the fact that brushite *in vivo* tends to precipitate as insoluble HA slowing its replacement by bone (Tamimi (2009)). However, monetite is slightly less soluble and appears not to transform to HA. Combined with the fact that monetite is osteoconductive and resorbable *in vivo* (Gbureck (2007); Tamimi (2007); Habibovic (2008)), it forms an excellent candidate as a bone graft substitute or as a porous matrix for bone tissue engineering.

One current disadvantage associated with monetite scaffolds is that they cannot be formed *in situ* as the process of making monetite requires the chemical conversion of a solid calcium phosphate phase by a hydrothermal process which requires high temperatures, often in excess of 100°C. Therefore, previously, monetite could only be used in the form of a pre-formed scaffold.

Summary of the Invention

The inventors have devised a new method for obtaining monetite from chemical formulations typical of hydraulic cements. This method allows monetite to be obtained as the final product of the setting reaction of a hydraulic cement and thus allows the use of monetite as a mouldable and injectable paste to treat bone defects, as well as preformed scaffold or a cell seeded scaffold since it does not require the use of relatively high temperatures.

Accordingly, in a first aspect, the invention provides a method of making monetite comprising mixing an aqueous phase and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and forming monetite.

In some embodiments, the invention provides a method of making monetite comprising mixing an aqueous phase and a solid phase comprising a basic calcium salt and an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and forming monetite.

In other embodiments, the invention provides a method of making monetite comprising mixing an aqueous phase comprising an acid and a retardant with a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and forming monetite.

The invention also provides a composition for forming monetite comprising a basic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

In some embodiments, the invention provides a composition for forming monetite comprising a basic calcium salt, an acidic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

The invention additionally provides a cement for forming monetite comprising: an aqueous phase; and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

In some embodiment, the invention provides a cement for forming monetite comprising: an aqueous phase; and a solid phase comprising a basic calcium salt and an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

In other embodiments, the invention provides a cement for forming monetite comprising: an aqueous phase comprising an acid and a retardant; and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

Further, the invention provides a composition or a cement as described above for use in therapy or surgery. For example, the composition and the cement may generally be used in bone regeneration and, in particular, may be used in traumatology surgery, maxillofacial surgery, periodontal surgery, orthognathic surgery, oral surgery, neurosurgery, palatine fissure treatment, periodontal treatment, treatment of dental conducts, treatment of osteoporotic bone, and alveolar regeneration and horizontal alveolar regeneration, or bone regeneration.

In another aspect, the invention provides a kit for forming monetite comprising: a basic calcium salt; and an inorganic salt comprising a mono or divalent metal cation and a halide anion.

In some embodiments, the invention provides a kit for forming monetite comprising: a basic calcium salt; an acidic calcium salt; and an inorganic salt comprising a mono or divalent metal cation and a halide anion.

In other embodiments, the invention provides a kit for forming monetite comprising: a basic calcium salt; an acid; a retardant; and an inorganic salt comprising a mono or divalent metal cation and a halide anion.

Additionally, the invention provides a method of treatment comprising administering to a subject an effective amount of a composition comprising a basic calcium salt, and an inorganic salt comprising a

mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

In some embodiments, the invention provides a method of treatment comprising administering to a subject an effective amount of a composition comprising a basic calcium salt, an acidic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

The invention also provides a method of treatment comprising administering to a subject an effective amount of a cement comprising an aqueous phase and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

In some embodiments, the invention provides a method of treatment comprising administering to a subject an effective amount of a cement comprising an aqueous phase and a solid phase comprising a basic calcium salt and an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

In other embodiments, the invention provides a method of treatment comprising administering to a subject an effective amount of a cement comprising: an aqueous phase comprising an acid and a retardant; and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

Further features and aspects of the invention will be described in more detail below.

Detailed Description of the Invention

As indicated above, the invention provides a method of making monetite comprising: mixing a solid phase comprising a basic calcium salt with an aqueous phase, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and forming monetite.

In this method, the basic calcium salt and the acidic calcium salt contained in the solid phase react together to form monetite. The method of the invention allows the formation of monetite without the method involving the use of a hydrothermal step requiring high temperatures to produce the monetite, as with previously known methods. The method of the invention allows monetite to be formed at room temperature and under physiological conditions. Surprisingly, it has been found that the presence of the inorganic salt affects the reaction kinetics thereby allowing the production of monetite rather than

brushite. This means that monetite can be formed in situ, for example, in a bone cavity in a subject. Monetite is preferred to brushite since it has better physical properties and is osteoconductive and resorbable *in vivo*.

The solid phase comprises the components which react together to form the monetite. Preferably, all the components of the solid phase (including any optional additional components) are fully mixed prior to being mixed with the aqueous phase.

In the embodiment in which the solid phase comprises a basic calcium salt and an acidic calcium salt, the solid phase comprises a basic calcium salt and an acidic calcium salt which, when mixed with an aqueous phase, react together to form the monetite. The basic calcium salt may be a basic calcium phosphate. For example, the basic calcium salt may be selected from α-tricalcium phosphate, β-tricalcium phosphate, hydroxyapatite, tetracalcium phosphate, octacalcium phosphate and calcium oxide. In one embodiment, the basic calcium salt is β-tricalcium phosphate. The acidic calcium salt may be an acidic calcium phosphate. For example, the acidic calcium salt may be selected from monocalcium phosphate anhydrous or monocalcium phosphate monohydrate. In one embodiment, the acidic calcium salt is monocalcium phosphate monohydrate.

The ratio of the basic calcium salt to the acidic calcium salt in the solid phase may be any suitable ratio which allows the formation of monetite. The molar ratio of the basic calcium salt to the acidic calcium salt may be between 1:4 and 4:1, more preferably between 1:3 and 3:1, more preferably still between 1:2 and 2:1, even more preferably between 1.5:1 and 1:1.5 and, more preferably still between 1.2:1 and 1:1.2. In one embodiment, the molar ratio may be about 1:1.

In the embodiment in which the solid phase comprises a basic calcium salt, the solid phase comprises a basic calcium salt which, when mixed with an aqueous phase, reacts to form the monetite. The basic calcium salt may be a basic calcium phosphate. For example, the basic calcium salt may be selected from α-tricalcium phosphate, β-tricalcium phosphate, hydroxyapatite, tetracalcium phosphate, octacalcium phosphate and calcium oxide. In one embodiment, the basic calcium salt is β-tricalcium phosphate.

The aqueous phase can be any suitable aqueous liquid which, when mixed with the solid phase, causes the components in the solid phase to react together to form monetite. The aqueous phase may be water or an aqueous solution. The aqueous phase may comprise an acid such as glycolic, tartaric, citric, succinic, malic, lactic, or a salt thereof. In one embodiment, the aqueous phase comprises citric acid or a salt thereof such as monosodium citrate or trisodium citrate. The concentration of the acid or salt thereof may be between 0.3M and 4M, preferably between 0.4M and 3M, and more preferably still between 0.5M and 2M. In one embodiment, the concentration of the acid or salt thereof is between 0.5M and 1M.

In particular embodiments, if the aqueous phase comprises an acid, the acid is preferably a weak acid. Preferably, the aqueous phase does not comprise a strong acid such as hydrochloric or sulphuric acid. A strong acid is defined as one having a $pK_a < -1.74$. Further, the aqueous phase preferably should not comprise phosphoric acid.

Without wishing to be held to any particular theory, it is thought that the use of a strong acid, such as hydrochloric or sulphuric acid, or phosphoric acid causes the rapid dissolution of one or both of the calcium salts. In some embodiments, this can cause the pH of the solution to drop to a relatively low level. The rapid dissolution and/or relatively low pH of the solution favours fast reaction kinetics in terms of crystal growth which causes the formation of brushite instead of monetite. The aqueous phase should favour slow crystal growth kinetics which allow the formation of monetite rather than brushite.

Alternatively, in some embodiments, the aqueous phase may comprise a strong acid such as phosphoric acid. When a strong acid is present, the aqueous phase preferably comprises a retardant such as citric acid. In such embodiments, it is thought that the conversion into monetite happens because the pH of the components during setting is very acidic (as low as 1.6) but the rate of the setting reaction is slowed down by the presence of the retardant. Therefore, this results in the formation of monetite rather than brushite.

In particular, in the embodiment in which the solid phase comprises a basic calcium salt, the aqueous phase preferably comprises an acid. More preferably, the aqueous phase comprises a strong acid. Preferably, the strong acid is phosphoric acid. Preferably, the concentration of the acid is between 0.3M and 4M, more preferably between 0.4M and 3M, even more preferably between 0.5M and 2M and more preferably still between 0.5M and 1.5M. In one embodiment, the concentration of the acid is about 1M and is preferably phosphoric acid.

The aqueous phase can act as a retardant solution. When the solid phase and aqueous phase are mixed together, the composition can harden to form a solid cement very quickly. This does not allow enough surgical handling time. The addition of salt solutions such as citric acid or pyrophosphate ions to the aqueous phase retards the precipitation reaction and provides an increased handling time. The slowing down of the reaction kinetics using a retardant may also favour the formation of monetite rather than brushite.

In the embodiment in which the solid phase comprises a basic calcium salt, the aqueous phase preferably comprises a retardant. A retardant is a compound which increases the time it takes for the final product to form. A retardant increases the setting time of the cement, i.e. it is a setting retardant. Any suitable retardant can be used. In one embodiment, aconitic acid, citric acid or a salt thereof is used as a retardant. Preferably, citric acid or a salt thereof is used as the retardant. More preferably,

citric acid is used as a retardant. The concentration of the retardant may be between 0.3M and 4M, preferably between 0.3M and 3M, more preferably between 0.3M and 2M, even more preferably between 0.3M and 1M, and more preferably still between 0.3M and 0.7M. In one embodiment, the concentration of the retardant (e.g. citric acid) is about 0.5M.

The inorganic salt comprises a mono or divalent metal cation and a halide anion. Preferably, the mono or divalent metal cation is selected from a group 1 or 2 metal. In one embodiment, the metal cation is selected from sodium, potassium, magnesium, calcium and strontium. The metal cation may be a monovalent cation. The metal cation may be selected from a group 1 metal such as sodium or potassium. The halide anion may be selected from fluoride, chloride or bromide. Preferably, the halide anion is chloride. In some embodiments, the inorganic salt is selected from sodium chloride, potassium chloride, magnesium chloride, calcium chloride and strontium chloride. More preferably, the inorganic salt is selected from sodium chloride and potassium chloride. In one embodiment, the inorganic salt is sodium chloride. In one embodiment, the inorganic salt is not strontium chloride.

The inorganic salt may be contained in the solid phase, aqueous phase or both. In one embodiment, the inorganic salt is contained in the solid phase. In another embodiment, the inorganic salt is contained in the aqueous phase. In yet another embodiment, the inorganic salt is contained in both the aqueous phase and the solid phase.

In the embodiment in which the solid phase comprises a basic calcium salt and an acidic calcium salt, when the inorganic salt is contained in the solid phase, the weight ratio of the inorganic salt relative to the total weight of the basic calcium salt and acidic calcium salt combined may be between 1:199 and 4:1. In some embodiments, the ratio of the inorganic salt relative to the total weight of the basic calcium salt and acidic calcium salt combined may be between 1:19 and 7:3, between 1:2 and 2:1, between 4:6 and 6:4, or about 1:1. In one embodiment, the inorganic salt makes up at least about 5% by weight of the composition comprising the calcium salts and inorganic salt. Preferably, the composition comprises at least about 10% by weight inorganic salt. In some embodiments, the composition may comprise at least about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or at least about 70% by weight inorganic salt.

In the embodiment in which the solid phase comprises a basic calcium salt, when the inorganic salt is contained in the solid phase, the weight ratio of the inorganic salt relative to the weight of the basic calcium salt may be between 1:199 and 4:1. In some embodiments, the ratio of the inorganic salt relative to the weight of the basic calcium salt may be between 1:19 and 7:3, between 1:2 and 2:1, between 1:1 and 1:2, or about 0.6:1. In one embodiment, the inorganic salt makes up at least about 5% by weight of the composition comprising the calcium salt and inorganic salt. Preferably, the composition comprises at least about 10% by weight inorganic salt. In some embodiments, the

composition may comprise at least about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or at least about 70% by weight inorganic salt.

The advantage of having the inorganic salt in the solid phase is that it causes formation of pores in the monetite. The degree of porosity of the monetite can be manipulated by varying the salt content of the solid phase. The salt crystals are incorporated into the structure of the monetite during its formation and can subsequently be dissolved out of the monetite to leave pores. In this regard, the size of the particles of the inorganic salt will have an effect on the size of the pores that are formed in the monetite. In some embodiments, the salt is in the form of spherical crystals having a diameter ranging between 10 μm and 900 μm. The salt crystals may have a diameter of between 50 μm and 250 μm or between 100 μm and 200 μm. The presence of interconnecting pores in the monetite matrix advantageously allows cell ingrowth, proliferation and differentiation. Pores, introduced in the microstructure of the monetite, create an osteoconductive matrix which allows cell adhesion, proliferation and bone growth. The size of the pores required depend on cell types used for seeding.

When the inorganic salt is contained in the aqueous phase (i.e. the inorganic salt is in solution in the aqueous phase), the concentration of the inorganic salt may be at least about 1M. In some embodiments, the concentration of the inorganic salt may be at least about 1.5M, 2M, 2.5M, 3M, 3.5M, 4M, 4.5M, or at least about 5M. The concentration may be up to a point where the aqueous phase is saturated with the inorganic salt. The actual concentration of the saturated salt solution will depend on the identity of the inorganic salt. Preferably, the aqueous phase is saturated with respect to the inorganic salt. In one embodiment, a super saturated sodium chloride solution may be used for the conversion to monetite.

Without wishing to be held to any particular theory, it is thought that having a relatively high concentration of inorganic salt in the reaction solution, whether the inorganic salt is initially contained in the aqueous phase, solid phase or both, favours slow reaction kinetics in terms of crystal growth which allows the formation of monetite rather than brushite.

When the inorganic salt is contained in the solid phase and aqueous phase, the ratio and concentration of the salt in the two phases may be as described above with relation to the solid phase and aqueous phase.

The solid phase (P) and aqueous phase (L) can be mixed in any suitable way so that formation of monetite takes place. Preferably, the basic calcium salt and acidic calcium salt are mixed together to form the solid phase before being mixed with the aqueous phase. The ratio of the solid phase to the aqueous phase when mixed together may be between 4:1 to 1:1 (P:L). These are weight/volume ratios. The viscosity and thus the injectability can be varied by changing P:L ratios.

In the embodiment in which the solid phase comprises a basic calcium salt, the ratio of the solid phase to the aqueous phase when mixed together may be between 4:1 to 1:1 (P:L). Preferably, the ratio of the solid phase to the liquid phase is about 2:1, wherein the liquid phase preferably comprises phosphoric acid.

The method may be carried out under any suitable conditions which allow the formation of monetite. Preferably, the method is carried out at less than 60°C. The method may be carried out at less than 55°C, less than 50°C, less than 45°C, less than 40°C, less than 35°C, less than 30°C, less than 25°C or less than 20°C. This means that monetite formation takes place at less than 60°C, less than 55°C, less than 50°C, less than 45°C, less than 40°C, less than 35°C, less than 30°C, less than 25°C or less than 20°C. Preferably, the method is carried out at above 0°C. More preferably, the method is carried out at above 10°C or above 15°C. In some embodiments, monetite formation may take place at about body temperature (about 37°C) and/or at about room temperature (20-25°C). Preferably, the method is carried out at ambient temperature. In one embodiment, the method does not involve a step of heating the components to form monetite. In other embodiments, the components are not heated above 60°C, above 55°C, above 50°C, above 45°C, above 40°C, above 35°C, above 30°C, above 25°C or are not heated above 20°C. It is feasible that the components of the method could be heated up to a temperature of about 50-55°C and could still be used *in situ* in a subject, since it may take 30 seconds or more at such a temperature to cause burns. Therefore, an elevated temperature of up to about 50-55°C could be used in the method for a short period of time without causing harm to the subject.

The method may be carried out at a relative humidity of 30% to 100%.

The method should be carried out at a pH which allows the formation of monetite. Preferably, the method should be carried out at a pH of less than or equal to 5 or, more preferably, less than or equal to 4.2. In certain embodiments, the method may be carried out at a pH of less than 4.2, 4, 3.5, 3, 2.5 or less than 2. In some embodiments, the method is preferably carried out at a pH of at least about 1, 1.5, 2, 2.5, 3 or at least about 3.5.

The method is carried out for a period of time which allows the formation of monetite to take place. When referring to cements used to form monetite, the time that it takes to form the monetite is referred to as the setting time. Setting of the cement starts to occur once the solid phase has been mixed with the aqueous phase. Depending on the inorganic salt that is used in the method, the amount of time that it takes for the monetite to form and for the cement to set varies. Further, the temperature at which the method is carried out may cause a variation in the setting time, i.e. the time it takes for the formation of monetite. Preferably, when measured at about 37°C, monetite formation takes place in less than 36 hours, less than 30 hours, less than 24 hours, less than 18 hours, less than 12 hours, less than 6 hours, less than 4 hours, less than 3 hours, less than 2 hours, less than 1 hour, or less than 30 mins. Preferably, when measured at about 23°C, monetite formation takes place in less than 36 hours, less than 30 hours,

less than 24 hours, less than 18 hours, less than 12 hours, less than 6 hours, less than 4 hours, less than 3 hours, less than 2 hours, less than 1 hour, less than 45 mins, or less than 35 mins.

As indicated above, the identity of the inorganic salt used in the method may affect the amount of time that it takes for the monetite to form. Therefore, the period of time for which the basic calcium salt and the acidic calcium salt contained in the solid phase must be left, once mixed with the aqueous phase, before they react together to form monetite varies depending on the inorganic salt. When the salt is sodium chloride, monetite formation occurs relatively quickly so the components of the solid phase only need to be left between about 2 and about 60 minutes before monetite formation takes place. When the salt is potassium chloride, calcium chloride or magnesium chloride the components of the solid phase are preferably left for more than about 24 hours to allow monetite formation to take place.

The method of the invention allows production of monetite from a basic calcium salt and an acidic calcium salt, or just a basic calcium salt. Preferably, conversion of the calcium salt(s) to monetite is at least 40%. This means that at least 40% of the final product is monetite relative to the total amount of calcium compounds in the final product. In other embodiments, conversion of the calcium salt(s) to monetite is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or about 100%.

When the solid and liquid phases are mixed, a cement paste is formed that hardens, solidifies and then sets to form a material which consists mainly of monetite. When the monetite is formed, i.e. when it sets, it will be formed into a solid. In certain embodiments, the monetite may form into a solid matrix. In some embodiments, the components can be put into moulds prior to setting so that the monetite forms in a particular shape. The monetite may be formed into blocks, granules, rods, sheets, sponges, pellets or other shapes. Monetite blocks, once formed, may subsequently be pulverised to form granules, or their shape may be adapted to the intended use by fragmentation, abrasion or filing. Cell seeded constructs can be used for bone tissue engineering.

The solid phase can also comprise further components as long as the solid phase, when mixed with the aqueous phase, is still capable of reacting to form monetite. For example, the solid phase may further comprise antibiotics or growth factors.

In the method, further additives can be mixed with the solid phase and/or the aqueous phase. For example, additives may be included in the solid phase that increase the porosity of the resulting matrix through the liberation of gas during the setting process, for example, calcium carbonate, calcium bicarbonate, sodium bicarbonate, hydrogen peroxide, or other salts. Furthermore, porosity of the resulting matrix can also be increased by incorporating soluble additives when the solid phase is mixed with the aqueous phase. These soluble additives are incorporated into the monetite matrix and can subsequently be dissolved, thereby creating pores where the additives used to present in the matrix.

Examples of such soluble additives include organic or inorganic salts, sugars, sugar alcohols, amino acids, proteins, polysaccharides or water soluble polymers.

The solid phase may also incorporate additives to control the rheology of the components. These additives may comprise chondroitin 4-sulphate, chondroitin 6-sulphate, a silica gel, a silica gel with chondroitin 4-sulphate, a silica gel with chondroitin 6-sulphate, strontium chloride, strontium renalate, any salt containing strontium, sodium pyrophosphate, calcium pyrophosphate, and/or any salt or acid containing pyrophosphate groups. Biocompatible agents such as collagen, chitosan, albumin, fibronectin, hyaluronic acid, hyaluronate salts, dextran, alginate, xanthan gum or celluloses can also be incorporated to control the rheology of the components. In one embodiment, the solid phase can incorporate high molecular weight hyaluronic acid, chitosan, hydroxypropyl methyl cellulose, polyethylene glycol (PEG) or polyacrylic acid.

The aqueous phase can also contain additives to control the cohesion of the components of the solid phase such as chondroitin 4-sulphate, chondroitin 6-sulphate, silica gel, or a combination of silica gel with chondroitin 4-sulphate or chondroitin 6-sulphate. The concentration of the chondroitin 4-sulphate and the chondroitin 6-sulphate in the aqueous phase may be between 1 and 6% while the concentration of the silica gel can be between 1 and 15g/L.

The monetite matrices, once formed, can be combined with bioactive agents that favour the bone regeneration process such as for example, growth factors, hormones, polysaccharides, cells, proteins, peptides, anti-infectives, analgesics, anti-inflammatory agents, antibiotics, antigens, MMP inhibitors or any combination thereof. Incorporation of such agents can be carried out by means of adsorption or immersion in solutions containing the bioactive agent. The growth factors may include platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), bone morphogenic proteins (BMP), transforming growth factor-beta-1 (TGF-β-1), growth hormone (GH), insulin like growth factor-1 (IGF1), insulin like growth factor-2 (IGF2), fibroblast growth factor (FGF) or any combination thereof.

The proteins may include collagen, fibronectin, albumin or any of their combinations thereof. Furthermore undefined media can also be incorporated to promote bone regeneration, such as blood, serum or plasma.

To improve the stability of the bioactives adsorbed to the monetite matrix, stabilising agents such as trehalose, sucrose, raffinose, manitol, albumin or collagen can be added to the solution containing the bioactive. Among the bioactive agents that can be incorporated to the monetite is strontium. The addition of strontium, or any of its salts and in concentrations up to 10%, permits that blocks, granules, rods, sponges or pellets can be used in the treatment of osteoporosis.

The invention also relates to compositions, cements, the use of these compositions and cements, kits and methods of treatment which will be described in detail below. The skilled person will appreciate that the description above of the method of the invention is equally applicable to all the other aspects of the invention. Therefore, if a particular feature has only been described with respect to the method of the invention, a skilled person should appreciate that this feature also applies to the other aspects of the invention even if it has not been explicitly described in relation to the other aspects. In fact, a skilled person will appreciate that wherever a feature is described, it will be applicable to all the other aspects of the invention.

The invention also provides a composition for forming monetite comprising a basic calcium salt, optionally an acidic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

Preferred features of the composition are as described above with regard to the method of the invention. For example, the basic calcium salt may be β-tricalcium phosphate. If present, the acidic calcium salt may be monocalcium phosphate monohydrate. The inorganic salt may be selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride. The composition can be mixed with an aqueous phase to form a cement paste which will then harden and set, forming solid monetite.

The invention additionally provides a cement for forming monetite comprising: an aqueous phase; and a solid phase comprising a basic calcium salt and optionally an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

Preferred features of the cement are as described above with regard to the method of the invention. For example, the basic calcium salt may be β-tricalcium phosphate. If present, the acidic calcium salt may be monocalcium phosphate monohydrate. The inorganic salt may be selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride. The aqueous phase may be water or may comprise citric acid, monosodium citrate or trisodium citrate. Since the cement comprises an aqueous phase, it will harden and set, forming solid monetite.

In another aspect, the invention provides a kit for forming monetite comprising: a basic calcium salt; optionally an acidic calcium salt; and an inorganic salt comprising a mono or divalent metal cation and a halide anion. Preferred features of the kit are as described above with regard to the method of the invention. For example, the basic calcium salt may be β-tricalcium phosphate. The acidic calcium salt, if present, may be monocalcium phosphate monohydrate. The inorganic salt may be selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride. The kit of the

invention can be used to prepare the composition of the invention or the cement of the invention as described above.

The kit may further comprise a further component which can be added to an aqueous liquid to form an aqueous phase for use with the other components of the kit. For example, the kit may further comprise citric acid, monosodium citrate or trisodium citrate.

Further, the invention provides a composition or a cement as described above for use in surgery or therapy. For example, the composition and the cement may generally be used in bone regeneration and, in particular, may be used in traumatology surgery, maxillofacial surgery, periodontal surgery, orthognathic surgery, oral surgery, neurosurgery, palatine fissure treatment, periodontal treatment, treatment of dental conducts, treatment of osteoporotic bone, and alveolar regeneration and horizontal alveolar regeneration, or bone regeneration. Generally, the composition and the cement are used in bone regeneration in non-load bearing bones.

Additionally, the invention provides a method of treatment comprising administering to a subject an effective amount of a composition comprising a basic calcium salt, optionally an acidic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

In this method of treatment, the composition can be administered in any suitable way. For example, the composition may be administered into, for example, a bone cavity in which it is desired to promote bone regeneration. The inorganic salt in the composition will have been incorporated into the monetite, therefore, once the inorganic salt dissolves in the aqueous environment, a porous monetite matrix will remain in the bone cavity. This porous monetite matrix will form a scaffold on which bone regeneration can take place.

The method of treatment can further comprise administering an aqueous phase. Preferred properties of the aqueous phase are as described above.

The invention also provides a method of treatment comprising administering to a subject an effective amount of an aqueous phase and a solid phase comprising a basic calcium salt and optionally an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein monetite is formed.

The solid phase and the aqueous phase can be administered concurrently or sequentially. Preferably the solid phase and the aqueous phase are administered concurrently in the form of a cement paste, i.e. a composition in which the solid phase and aqueous phase have been mixed. Accordingly, in a particular embodiment, the invention provides a method of treatment comprising administering to a subject an

effective amount of a cement comprising an aqueous phase and a solid phase comprising a basic calcium salt and optionally an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

In the above method, the components of the solid phase and the aqueous phase are mixed to form a cement paste which is able to harden and set to form solid monetite. Before the cement paste has started to harden or while it is hardening, it can be administered to a site of interest in a subject. For example, this might be a bone cavity in which it is desired to promote bone regeneration. The cement paste should be administered to the site of interest before it has set. This allows it to be moulded into a shape which is desired in the treatment process. The cement paste can be administered in any suitable way as long as the site of interest is filled or coated with the cement paste in an appropriate manner. For example, the cement paste may be injected into a bone cavity thus taking on the shape of the bone cavity itself. Once it has been administered, the cement paste will (continue to) harden until it has fully set at which point it will have converted into monetite. Once the monetite has formed at the site of interest, it can then form a scaffold for and promote bone regeneration. Preferably, the solid phase comprises the inorganic salt so that the salt is incorporated into the solid monetite thereby creating a porous monetite matrix. This is advantageous in terms of the promotion of bone regeneration.

Preferred features of the methods of treatment are as described above with regard to the method of forming monetite.

The subject to which the components for forming monetite are administered can be any subject having a skeletal system for which monetite can be used as a bone regeneration scaffold. The subject may be a vertebrate. Preferably, the subject is a mammal. In one embodiment, the subject is human.

The following examples and figures are provided by way of illustration and are not intended to be limiting of the present invention. The figures are as follows:

Figure 1 shows a comparison between the FTIR spectrum of commercial monetite (bottom spectrum) and the spectrum of cement made using NaCl crystals in the solid phase (top spectrum);

Figure 2 shows a comparison among FTIR spectrum of commercial monetite and those of the cements produced at different temperature (23 or 37°C) by using as liquid phase a monosodium citrate solution saturated with respect to NaCl crystals;

Figure 3 shows a comparison of the FTIR spectra of commercial monetite and cements made using different salts in the base formulation;

Figure 4 is an XRD pattern of calcium phosphate powders obtained by grinding cement made with NaCl crystals in the base formulation;

Figure 5 is a graph showing the results of a MTT test wherein the osteoblast like cells were exposed to 100 ml of elution fluid for 24 hrs; and

Figure 6 is a graph showing the results of a MTT test wherein the osteoblast like cells were exposed to 100 ml of elution fluid for 48 hrs.

Figure 7 shows the X-Ray patterns of monetite cements. The monetite references pattern (JCPDS00-009-0080) is shown on the bottom of the figure as spikes extending up from the y-axis.

Figure 8 shows the result of a cell proliferation test using the Alamar blue assay.

Figure 9 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 48 hours.

Figure 10 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 48 hours.

Figure 11 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 48 hours.

Figure 12 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 7 days.

Figure 13 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 7 days.

Figure 14 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts (HOB) after 7 days.

Figure 15 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts and shows that the cells were able to proliferate and to cover the scaffold surface.

Figure 16 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts and shows that the cells were able to proliferate and to cover the scaffold surface.

Figure 17 is a scanning electron microscopy image of a monetite cement seeded with human osteoblasts and shows that the cells were able to proliferate and to cover the scaffold surface.

Figure 18 is a graph showing the relative expression of osteogenic markers (Runx-2, alkaline phosphotase (ALP) and osteocalcin (OSC)) at 10 days after seeding.

Figure 19 is a graph showing the relative expression of osteogenic markers (Runx-2, alkaline phosphotase (ALP) and osteocalcin (OSC)) at 21 days after seeding.

Figure 20 shows the FTIR spectrum of commercial monetite (top) and that obtained using the method of the invention (bottom).

Figure 21 shows the FTIR spectra of CPCs made using a solution of NaCl (4mol/L and 5mol/L for cements A and B respectively) in distilled water as the liquid phase.

Figure 22 shows the FTIR spectra of CPCs made using a solution of 0.5M sodium citrate also containing 4mol/L (cement D ) or 5mol/L (cement E) NaCl as the liquid phase.

Figure 23 shows the FTIR spectra of CPCs made using a liquid phase containing NaCl (6mol/L) in distilled water (cement C) or in 0.5M sodium citrate solution (cement F).

Examples

Example 1

**Materials and Methods**

Monetite calcium phosphate cements have been produced by manually mixing a solid powder phase with a liquid phase. The solid phases consisting of:

- The compounds beta tricalcium phosphate ($\beta$-$Ca_3(PO_4)_2$) and monocalcium phosphate monohydrate ($Ca(H_2PO_4)_2 \cdot H_2O$) in molar ratio between 1:2 - 2:1; and
- Sodium chloride crystals (NaCl), in a quantity in weight ranging between 0.5 and 70% with respect to the weight of the total solid phase.

The liquid phase can be any of the following:

- A monosodium citrate solution ($C_6H_7O_7Na$) with molar concentration ranging between 0.5 and 2 mol/l where, the solution can optionally be saturated with respect to NaCl;
- A trisodium citrate solution ($C_6H_5O_7Na_3$) with molar concentration ranging between 0.5 and 2 mol/l where, the solution can optionally be saturated with respect to NaCl; or
- Water or a saturated solution of NaCl.

The ratio between the calcium phosphate powders and the liquid phase can be fixed at values ranging between 1.5 and 4.

Monetite cements were also prepared by using other salts such as potassium chloride, calcium chloride, magnesium chloride and strontium chloride with sodium chloride being the most effective. The use of a saturated solution of salt resulted in the formation of a monetite cement whereas the use of salt crystals in the solid phase yielded porous matrices.

In order to assess the extent of the setting reaction in terms of monetite formation, FTIR/ATR analysis using a Perkin Elmer Spectrum One FT-IR Spectrometer and XRD analysis were carried out. The setting time was evaluated by an indentation test using the Gilmore method and compressive mechanical strength was determined by uniaxial compressive test using a Universal testing machine (INSTRON 5569A) at a crosshead speed of 1 mm/min.

**Results**

**Setting Time**

The final setting time of the cements made using salts such as $MgCl_2$, $CaCl_2$ and KCl were found to be longer than 24 hrs. Different concentrations of solid salts were added to the reaction mixture and the conversion occurred at different concentrations for the salts. Higher concentrations yielded better results however the reaction kinetics were slow. Strontium chloride also yielded monetite with concentration of 50% by weight of the calcium phosphate powder (or calcium phosphate powder + salt) and can set within 2 minutes.

The test was carried out at normal laboratory atmosphere (20-23°C and 50-60% humidity) and at 37°C.

In table 1, the results of the setting time are shown when using NaCl in the base formulation of the cement. The NaCl crystals were added to the solid phase in a fraction weight equal to 50% with respect to the total weight of the solid phase (calcium phosphate powder + salt).

| Setting temperature | 23°C | 37°C |
|---|---|---|
| Initial setting time (min) | 12 ± 1 | 10 ± 1 |
| Final setting time (min) | 30 ± 2 | 20 ± 2 |

*Table1. Setting time of monetite cements made adding NaCl crystals in the solid phase. The results are shown as an average with the standard deviation of the measurements.*

Table 2 shows the setting time of monetite calcium phosphate cement obtained by mixing the calcium phosphate powders with an aqueous saturated solution of NaCl.

| Setting temperature | 23°C | 37°C |
|---|---|---|
| Initial setting time (min) | 8 ± 1 | 6 ± 1 |
| Final setting time (min) | 17 ± 2 | 16 ± 2 |

*Table 2. Setting time of monetite cements made by the addition NaCl solution to the reaction mix. The results are shown as an average with the standard deviation of the measurements.*

**FTIR and XRD analysis of the cement**

A comparison of the FTIR spectra of pure commercial monetite and the monetite cement obtained from the experiments of the addition of salt is shown in Figure 1. The composition of the experimental cement as seen from the spectra is identical and a complete conversion to monetite is observed. The FTIR spectra of monetite are characterized by the O-H in plane bending at 1404 and 1345 $cm^{-1}$. The main IR bands characteristic of the phosphate group can be detected at 1128, 1062 and 991 $cm^{-1}$ due to PO stretching modes of the $PO_3$ fragment. The P-O(H) stretching mode is observed at 1630 and 885$cm^{-1}$. The spectrum of the experimental cement shows all the characteristic peaks assigned to monetite [16]. The FTIR spectra of cements made using the saturated salt solution with monosodium citrate solution with the solid phase of the mixture of β-$Ca_3(PO_4)_2$ and $Ca(H_2PO_4)_2 \cdot H_2O$ are shown in Figure 2 and these show similar conversion to monetite. The reaction was carried out both at room temperature and 37°C and chemical conversion of the reagents into monetite resulted. Thus, the conversion of the brushite phase to monetite occurred using either saturated salt solution or crystalline salt. In Figure 3, a comparison of the FTIR spectra of the cements obtained by using different salts as $CaCl_2$, KCl and NaCl with the commercial monetite spectrum are presented. All salts yielded conversion to monetite but the kinetics were slower.

XRD analysis was performed in order to quantify the percentage of monetite in the cement made with NaCl crystals and the result is shown in Figure 4. The XRD result showed more than 95 percent degree of conversion of the cement components into monetite. Thus the introduction of sodium chloride resulted in obtaining monetite based cements via a simple and facile method under ambient conditions.

The compressive strength of the cements are shown in Tables 3 and 4, which show that the conversion to monetite either using solid sodium chloride or a saturated solution does not alter the mean compressive strengths.

| Sample | Mean σc [MPa] | S.D. σc [MPa] | Mean <E> [GPa] | S.D. E [GPa] |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| (F1)Brushite | 13.3 | 1.6 | 1.7 | 0.36 |
| (F2)Monetite | 10.7 | 0.9 | 1.3 | 0.34 |

*Table 3: Compressive strength of the cements*

*Table 4: Compressive strength of the cements; SD: standard deviation*

| Wt%<br>Salt | Mean<br>σc<br>[MPa] | S D<br>σc<br>[MPa] | Mean<br>E<br>[GPa] | S D<br>E<br>[GPa] |
|---|---|---|---|---|
| (F2)Monetite | 10.7 | 0.9 | 1.3 | 0.34 |
| (F3)Monetite 5% | 9.1 | 0.9 | 0.94 | 0.6 |

F1: β-TCP + MCMP + monosodium citrate solution giving rise to brushite.

F2: β-TCP + MCMP + sodium chloride crystals + monosodium citrate solution giving rise to monetite.

F3: β-TCP + MCMP + NaCl salt solution + monosodium citrate solution (0.5M) giving rise to monetite.

Finally cytocompatibility tests were carried out on the monetite cements obtained by this experimental method. In brief MTT test was performed in order to assess the cytotoxicity of the monetite scaffolds made by using NaCl salts in the base chemical formulation. The biological test is a colorimetric assay measuring the metabolic activity of enzymes that reduce the yellow tetrazolium MTT salts to formazan crystal giving a purple colour. The concentration of the formazan is determined by a spectrophotometric measure with optical density equal to 570nm. The reduction of the MTT, take place only when reductive enzymes are active, and therefore conversion is a measure of viable cells.

The graphs shown in Figures 5 and 6 are the results of MTT test wherein the osteoblast like cells were exposed to 100 ml of elution fluid for 24 hrs (Figure 5) and 48 hrs (Figure 6) respectively. Elution fluid was obtained by placing monetite samples in HOB medium for 24 and 72 hrs. The optical density measurements of the investigated materials were compared to those obtained for the non-toxic control (Thermanox) and toxic control (media with 10% of alcohol). As shown in the graphs, the material did not show any toxicity effect on the osteoblast viability. Monetite is a cytocompatible material and these tests confirm that the method of production does not alter any of the biological properties.

**X-Ray analysis**

Figure 7 shows the X-Ray patterns of a monetite cement made by using a solution of distilled water containing 6M of NaCl salt as the liquid phase (upper pattern) and a solution of monosodium citrate (0.5M). The solid phase for both cements was an equimolar mixture of β-TCP and MCPC powders. The result described by the lower pattern is for a cement in which the solid phase also contained an amount of NaCl equal to 60% by weight with respect to the calcium phosphate powders (The X-Ray pattern was detected once that the NaCl was dissolved in water by keeping the cement for 3 days in distilled water). The monetite references pattern (JCPDS00-009-0080) is shown on the bottom of the figure as spikes extending up from the y-axis.

The results show the main product of the setting reaction is monetite.

**Biological Evaluation**

**Cell proliferation: Alamar blue® assay**

The Alamar blue assay gives a quantification of the reducing environment of the cells. In fact, when cells are metabolising they maintain a reducing environment within their cytosol and this reduced state can be measured spectrophotometrically through the conversion of fluorometric/colorimetic REDOX indicators. This application focuses on quantification of the reduction of the intracellular environment by alamarBlue®. The reducing environment of the cells in the alamarBlue® assay is measured through the conversion of resazurin (oxidised form) to resorufin (reduced form). This results in colorimetric (absorbance) and fluorescence changes.

Figure 8 shows the result of the cell proliferation test. Human osteoblasts (HOB) were seeded on the surface of macroporous monetite cement scaffold (n=4) at a density of $1x10^5$ in 1 ml of normal growth media. The same amount of cells was seeded on the surface of plastic control scaffolds (thermanox). The assay was performed after 3, 7, 14 and 21 days from the seeding of the cells.

The results show that the cells seeded on monetite cements are able to maintain their viability and to proliferate over time.

**SEM Analysis**

Scanning electron microscopy was carried out using a Hitachi S-3500 machine and the microstructure recorded. Figures 9-17 show the images of the monetite cements seeded with human osteoblasts (HOB). In particular, Figures 9-14 show the ability of the cells to spread and adhere at the scaffold surface after 48 hrs (Figures 9-11) and 7 days (Figures 12-14) from the seeding. Figures 15-17 show that the cells were able to proliferate and to cover the scaffold surface.

**Osteogenic differentiation**

Osteogenic expression of human osteoblasts (HOB) was assessed by analysing the changes in expression of osteogenic markers (Runx-2, alkaline phosphotase (ALP) and osteocalcin (OSC)) at 10

(Figure 18) and 21 (Figure 19) days from the seeding by qRT-PCR procedure. The cells were seeded on the surface of monetite cements (n=4) at a density of $1x10^5$ in 1 ml of normal growth media and the same amount of cells were seeded on the top of thermanox discs (mono) used as a control. The results of the osteogenic expression were normalized with respect to the control.

**FTIR spectroscopy**

Figure 20 shows the FTIR spectrum of commercial monetite (on the top) and that obtained by using a solution of phosphoric acid ($H_3PO_4$, 2M) containing 6M of NaCl crystals and 0.5 M of citric acid as the liquid phase. The liquid phase was mixed with β-TCP powder in a powder to liquid ratio (R: P/L) equal to 2.

The results show that it is possible to obtain monetite as the main final product of the setting reaction using an oversaturated acid solution (in this case phosphoric acid containing citric acid as a retardant of the setting reaction) of sodium chloride salt as the liquid phase.

In particular, is important to note that the solid phase consists only of β-TCP powders.

**Conclusion**

The findings indicate that sodium chloride gives the most facile conversion of calcium phosphate powders into monetite. Potassium chloride also allows the conversion but the percentage conversion is greater at 37°C (body temperature) rather than at ambient temperature. Again calcium chloride and magnesium chloride allow some conversion but the setting of the cement requires more than 24 hours. Strontium chloride also showed some conversion to monetite. Other salts such as sodium bicarbonate were also tested (results not shown) but these did not allow conversion to monetite; brushite was produced instead.

Example 2

**Materials and Methods**

All calcium phosphate cements (CPCs) were produced by mixing equimolar quantity of β-TCP and MCPM powders. The liquid phase was distilled water or a retardant solution (which slows down the setting time) of monosodium citrate with a molar concentration equal to 0.5M.

In order to define the amount of NaCl crystals required to obtain monetite as the final product of the setting reaction, different cements were prepared. Cement A, B and C were obtained using distilled water with a molar concentration of NaCl equal to 4M, 5M and 6M respectively. Cements D, E and F were obtained with a liquid phase of 0.5M monosodium citrate solution and also containing 4M, 5M or 6M of NaCl respectively.

**Results**

Figures 21, 22 and 23 show the FITR spectra of these cements relative to pure monetite.

The results show that monetite can be obtained by using a solution of NaCl (5mol/L) in distilled water as liquid phase.

Monetite can also be obtained by using as liquid phase a solution of 0.5M sodium citrate containing 4 mol/L NaCl (cement D, Figure 22). In this case the spectrum is very similar to that of monetite except for the presence of some brushite peaks reported in figure as δ-OH and ν-OH mode.

In the case of a solution made with or without the retardant molecules of the setting time (sodium citrate), a molar concentration of NaCl equal to 6M is able to convert the reagents into monetite (see Figure 23).

Example 3

A Monetite cement can also be obtained by mixing a solid phase of β-tricalcium phosphate particles and NaCl crystals with a phosphoric acid solution containing citric acid as a retardant of the setting time. Further, a monetite cement can be obtained as reported above where the NaCl is present in both solid and liquid phase.

Monetite cements were made using the following formulations:

Formulation 1

Solid phase:

R0: (Ratio between β-TCP and phosphoric acid solution) = 1:1

R1: (Ratio between β-TCP and NaCl) = 1:0.6

Liquid phase:

Ortophosphoric acid solution (1M, 85%) containing 0.5M of citric acid.

Formulation 2

Solid phase:

R0: (Ratio between β-TCP and phosphoric acid solution) = 1:2

Liquid phase:

Ortophosphoric acid solution (2M) containing 6M of NaCl and 0.5M of citric acid.

An FITR spectrum of monetite produced using formulation 2 is shown in Figure 20.

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## Claims

1. A method of making monetite comprising:

mixing a solid phase comprising a basic calcium salt with an aqueous phase, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion; and

forming monetite.

2. The method of claim 1, wherein the basic calcium salt is β-tricalcium phosphate.

3. The method of claim 1 or claim 2, wherein the solid phase further comprises an acidic calcium salt.

4. The method of claim 3, wherein the acidic calcium salt is monocalcium phosphate monohydrate.

5. The method of claim 3 or claim 4, wherein the molar ratio of the basic calcium salt to the acidic calcium salt in the solid phase is between 1:2 and 2:1.

6. The method of claim 1, wherein the aqueous phase comprises phosphoric acid and a retardant.

7. The method of any preceding claim, wherein the aqueous phase comprises citric acid, monosodium citrate or trisodium citrate.

8. The method of claim 7, wherein the concentration of the citric acid, monosodium citrate or trisodium citrate is between 0.5M and 2M.

9. The method of any preceding claim, wherein the inorganic salt is selected from sodium chloride, potassium chloride, magnesium chloride, calcium chloride and strontium chloride.

10. The method of any preceding claim, wherein the inorganic salt is selected from sodium chloride and potassium chloride and, preferably, the inorganic salt is sodium chloride.

11. The method of any preceding claim, wherein the inorganic salt is contained in both the solid phase and the aqueous phase.

12. The method of any one of claims 1 to 10, wherein the inorganic salt is contained in the solid phase.

13. The method of claim 11 or claim 12, when dependent on claim 3, wherein the weight ratio of the inorganic salt relative to the basic calcium salt and acidic calcium salt combined is between 1:19 and 7:3 and, preferably, between 1:2 and 2:1.

14. The method of any one of claims 1 to 10, wherein the inorganic salt is contained in the aqueous phase.

15. The method of claim 11 or claim 14, wherein the concentration of the inorganic salt is above 4M.

16. The method of any preceding claim, wherein the ratio of the solid phase to the aqueous phase when mixed together may be between 1:1.5 and 1:4.

17. The method of any preceding claim, wherein the method is carried out at less than 50°C.

18. The method of any preceding claim, wherein the method is carried out at a pH of less than 4.2.

19. The method of any preceding claim, wherein conversion of the calcium salts to monetite is at least 90%.

20. The method of claim 1 comprising:

mixing a solid phase comprising β-tricalcium phosphate and monocalcium phosphate monohydrate with an aqueous phase, wherein the solid phase, aqueous phase or both comprises sodium chloride; and

forming monetite,

wherein the method is carried out at less than 40°C.

21. The method of claim 1 comprising:

mixing a solid phase comprising β-tricalcium phosphate with an aqueous phase comprising phosphoric acid and citric acid, wherein the solid phase, aqueous phase or both comprises sodium chloride; and

forming monetite,

wherein the method is carried out at less than 40°C.

22. A composition for forming monetite comprising a basic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

23. The composition of claim 22, wherein the basic calcium salt is β-tricalcium phosphate.

24. The composition of claim 22 or claim 23, wherein the composition further comprises an acidic calcium salt which is monocalcium phosphate monohydrate.

25. The composition of any one of claims 22 to 24, wherein the inorganic salt is selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride.

26. A cement for forming monetite comprising: an aqueous phase; and a solid phase comprising a basic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

27. The cement of claim 26, wherein the basic calcium salt is β-tricalcium phosphate.

28. The cement of claim 26 or claim 27, wherein the composition further comprises an acidic calcium salt which is monocalcium phosphate monohydrate.

29. The cement of any one of claims 26 to 28, wherein the inorganic salt is selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride.

30. The cement of any one of claims 26 to 29, wherein the aqueous phase is water or comprises citric acid, monosodium citrate or trisodium citrate.

31. The cement of claim 26 or claim 27, wherein the aqueous phase comprises phosphoric acid and citric acid.

32. A kit for forming monetite comprising: a basic calcium salt; and an inorganic salt comprising a mono or divalent metal cation and a halide anion.

33. The kit of claim 32, wherein the basic calcium salt is β-tricalcium phosphate.

34. The kit of claim 32 or claim 33, wherein the composition further comprises an acidic calcium salt which is monocalcium phosphate monohydrate.

35. The kit of any one of claims 32 to 34, wherein the inorganic salt is selected from sodium chloride, potassium chloride, magnesium chloride and calcium chloride.

36. The kit of any one of claims 32 to 35, further comprising citric acid, monosodium citrate or trisodium citrate.

37. The kit of claim 36, further comprising phosphoric acid.

38. The composition of any one of claims 22 to 25 or the cement of any one of claims 26 to 31 for use in surgery or therapy.

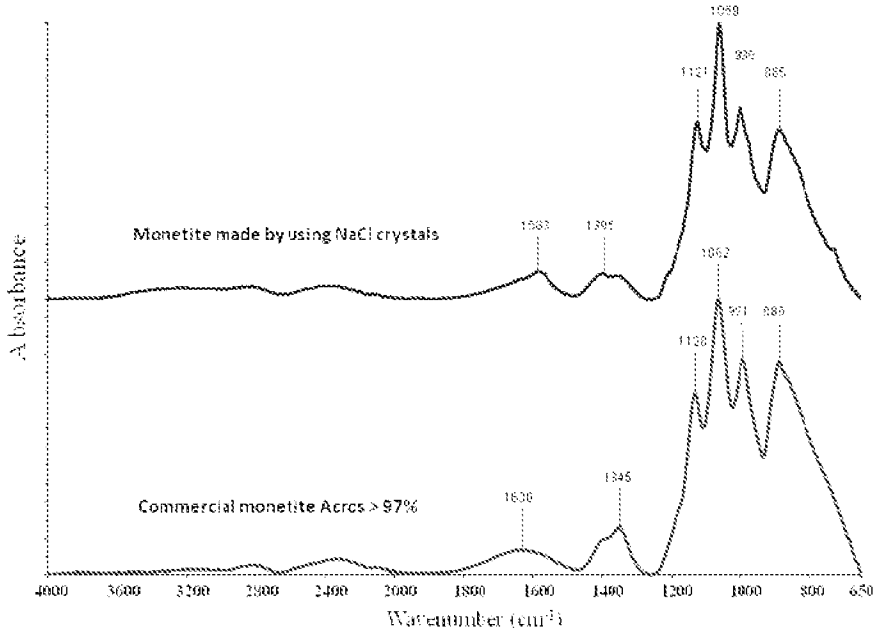
39. The composition of any one of claims 22 to 25 or the cement of any one of claims 26 to 31 for use in traumatology surgery, maxillofacial surgery, periodontal surgery, orthognathic surgery, oral surgery, neurosurgery, palatine fissure treatment, periodontal treatment, treatment of dental conducts, treatment of osteoporotic bone, and alveolar regeneration and horizontal alveolar regeneration, or bone regeneration.

40. A method of treatment comprising administering to a subject an effective amount of a composition comprising a basic calcium salt, optionally an acidic calcium salt, and an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein, when mixed with an aqueous phase, the composition sets to form monetite.

41. A method of treatment comprising administering to a subject an effective amount of an aqueous phase and a solid phase comprising a basic calcium salt and optionally an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein monetite is formed.

42. A method of treatment comprising administering to a subject an effective amount of a cement comprising: an aqueous phase; and a solid phase comprising a basic calcium salt and optionally an acidic calcium salt, wherein the solid phase, aqueous phase or both comprises an inorganic salt comprising a mono or divalent metal cation and a halide anion, and wherein the cement sets to form monetite.

## Figure 1




## Figure 2

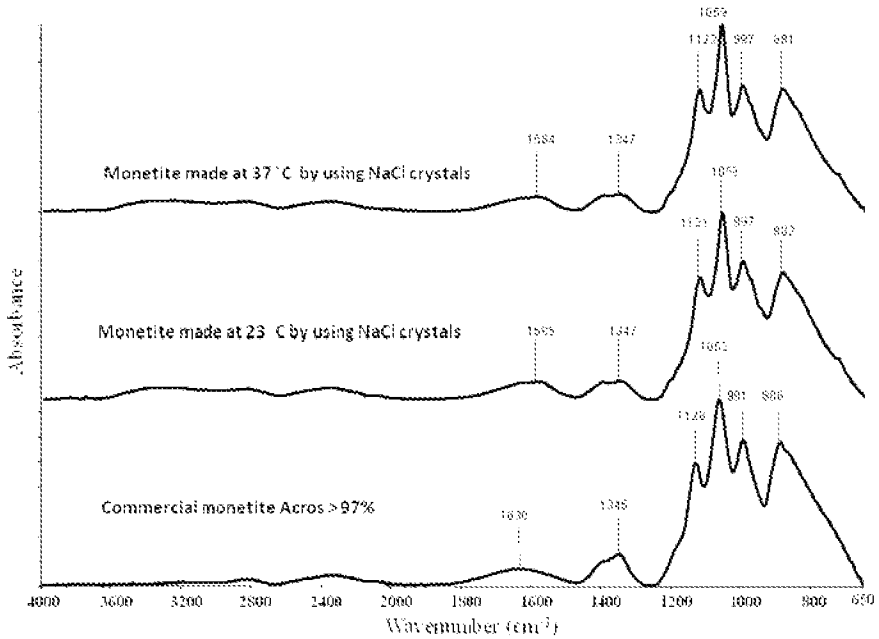


**Figure 3**

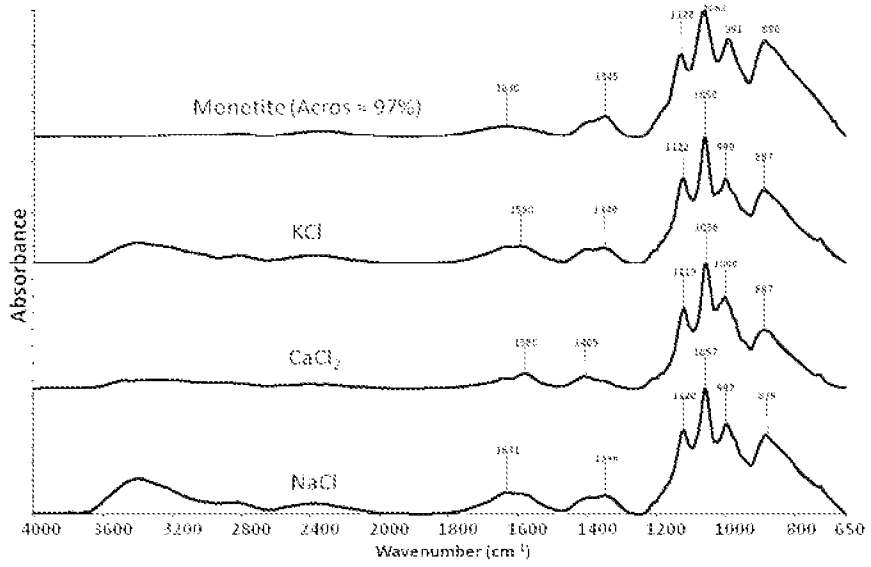



**Figure 4**

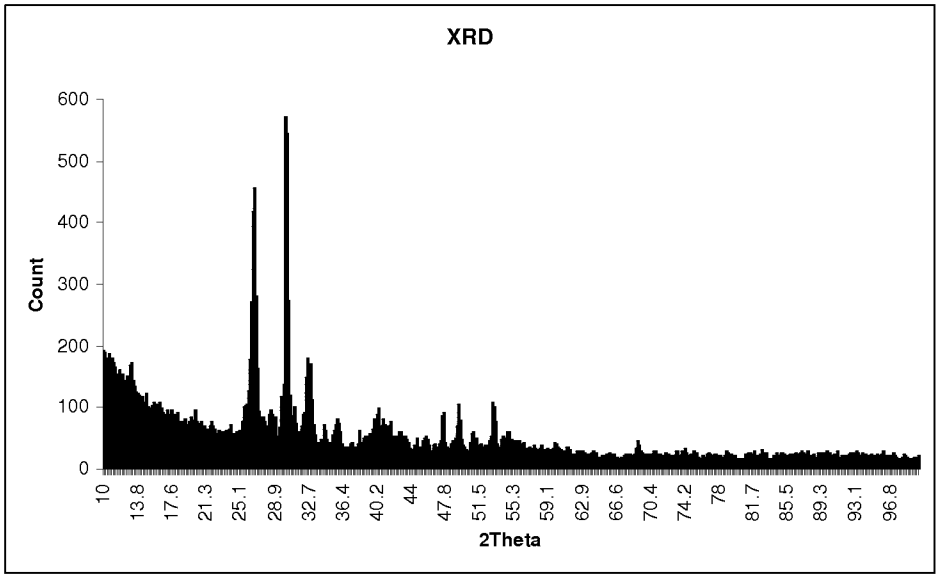


**Figure 5**

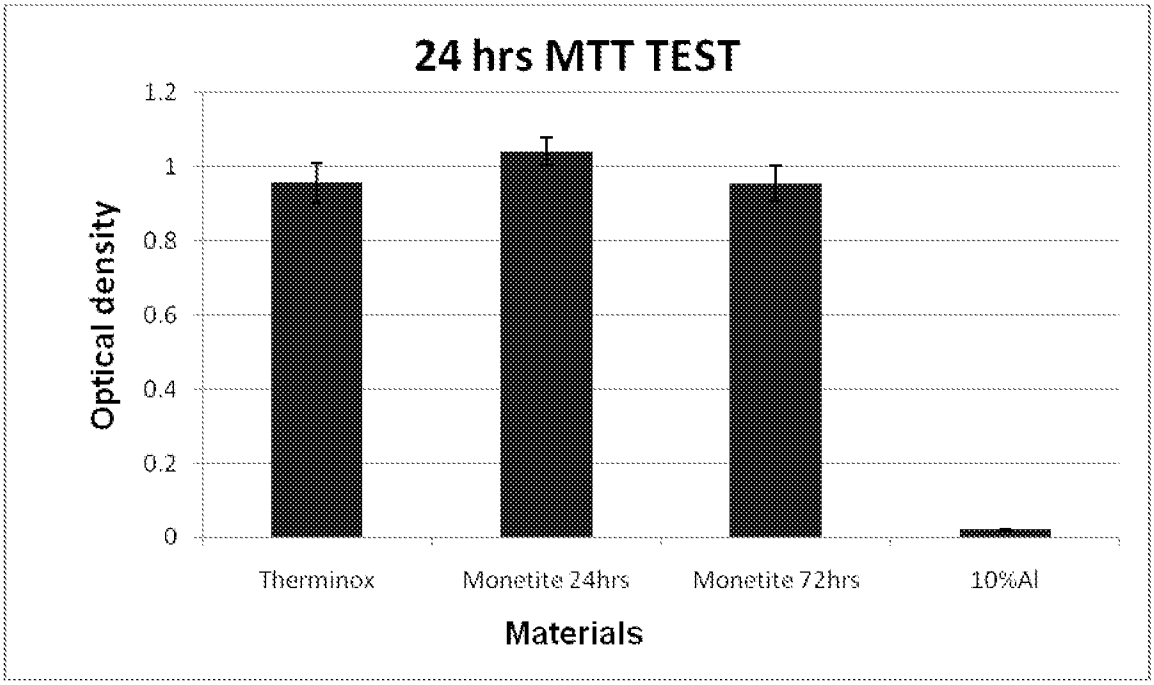



**Figure 6**

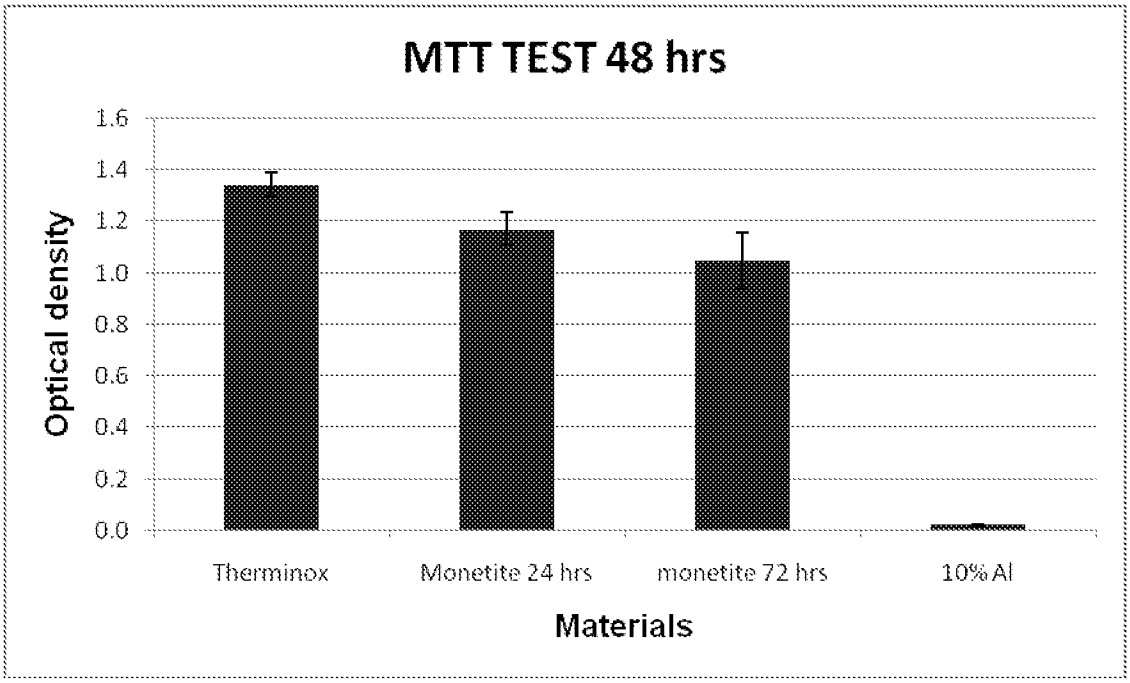


**Figure 7**

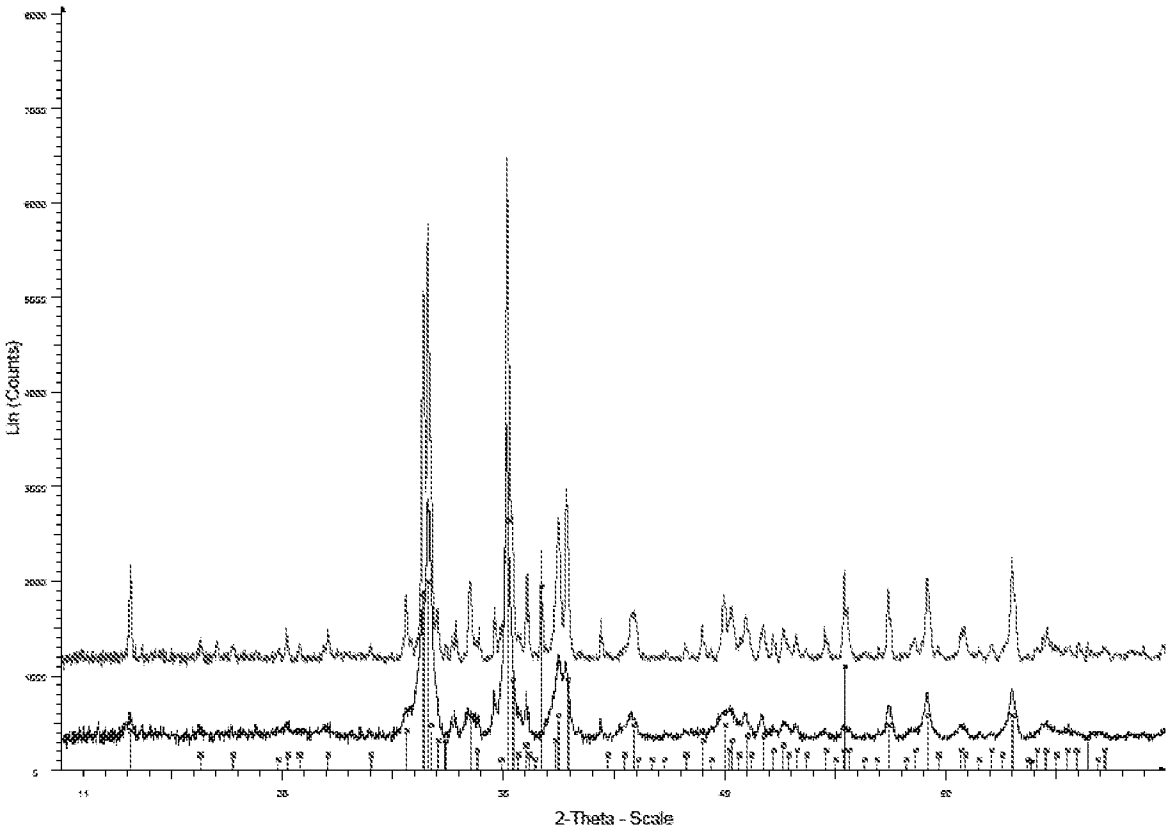



**Figure 8**

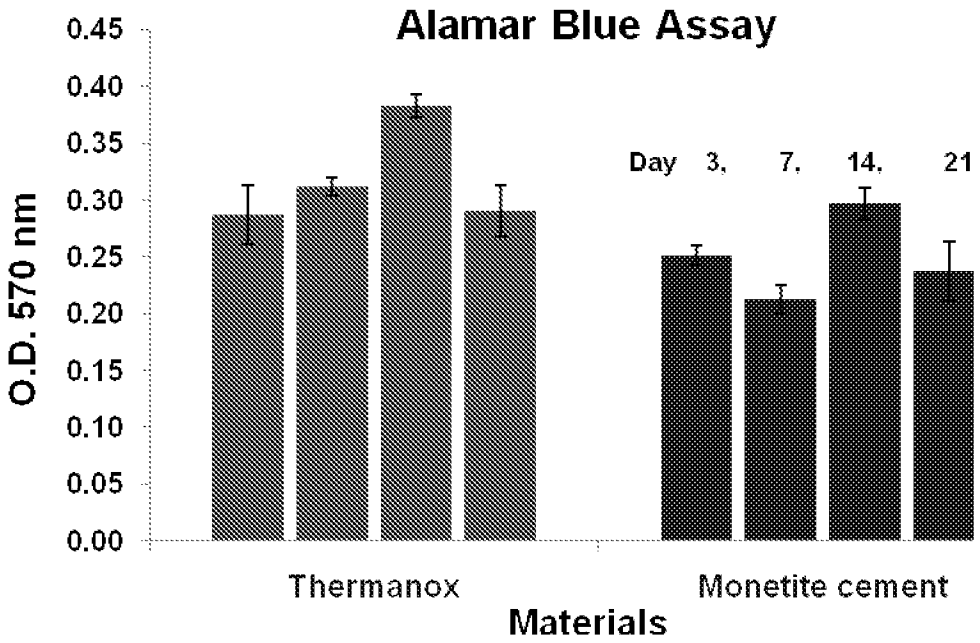


**Figure 9**

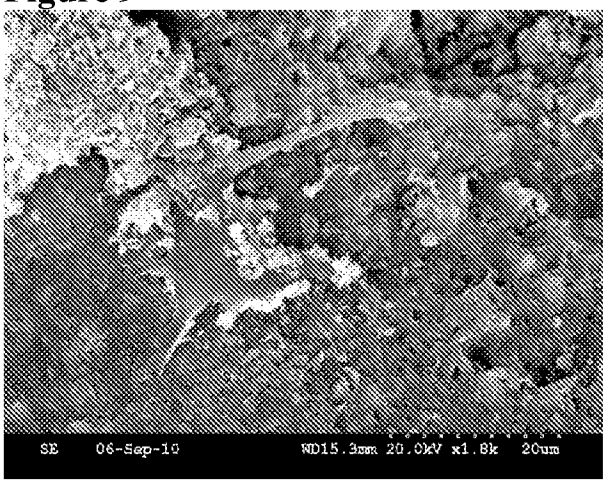



**Figure 10**

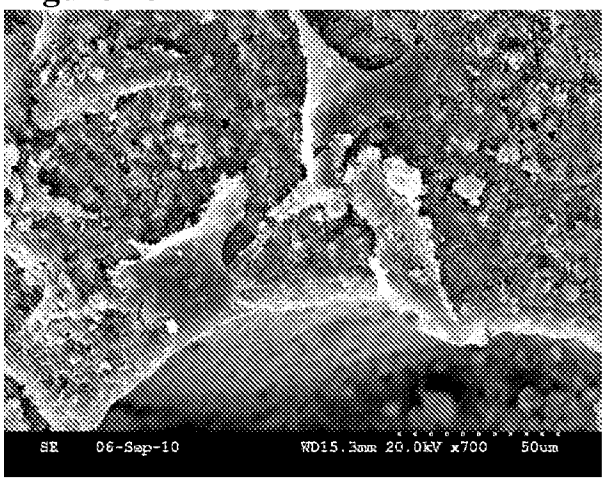



**Figure 11**

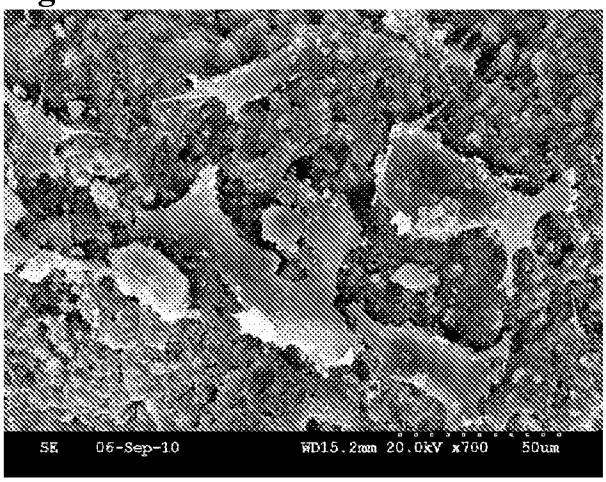



**Figure 12**

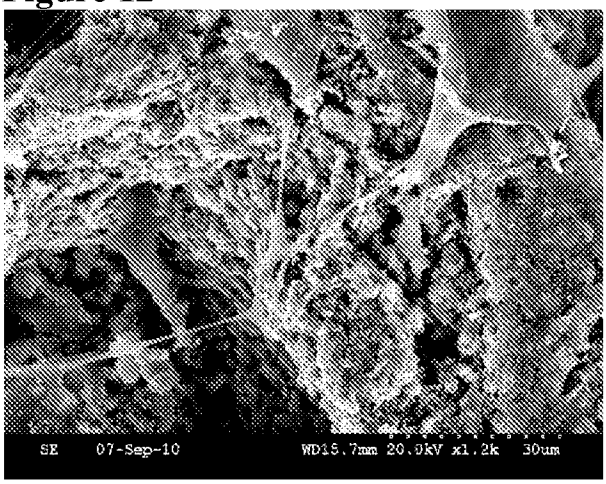



**Figure 13**

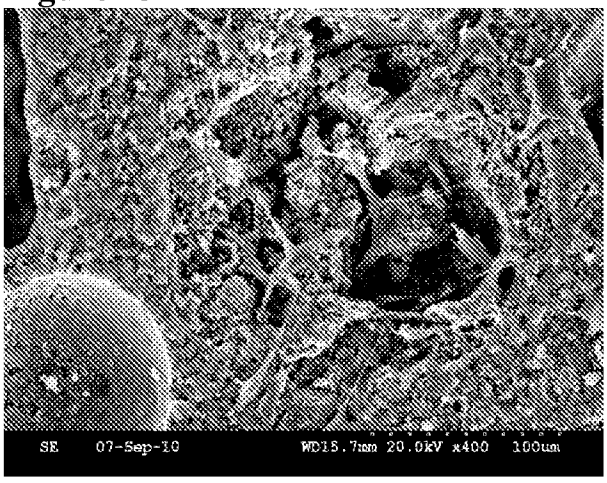



**Figure 14**

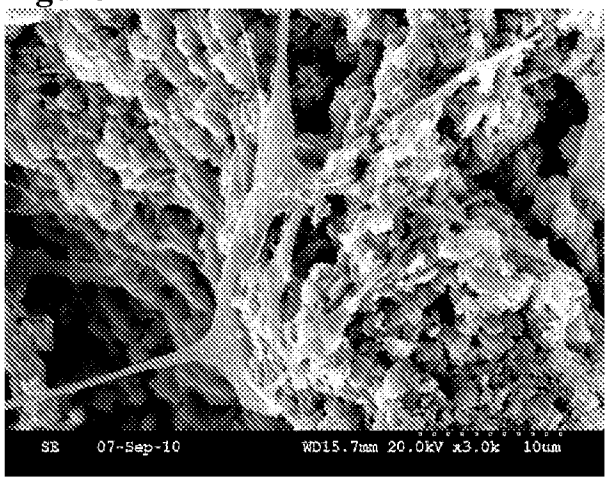


**Figure 15**

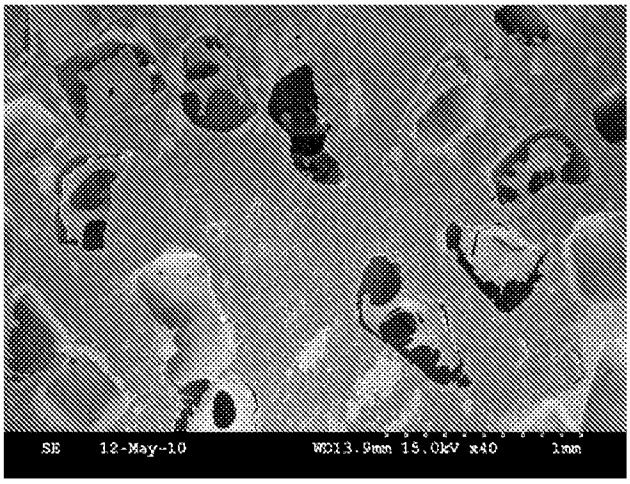



**Figure 16**

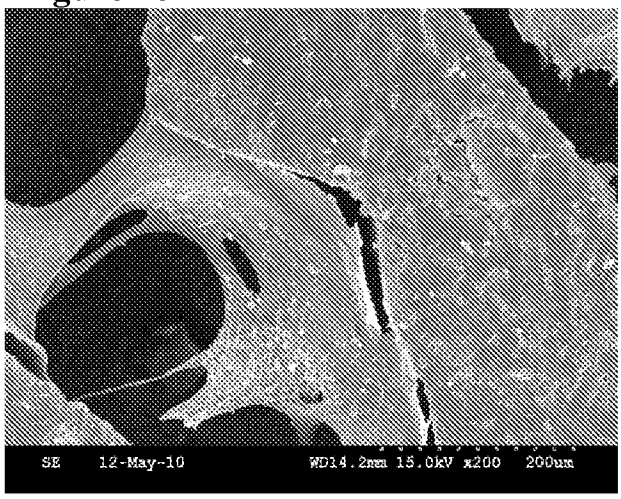



**Figure 17**

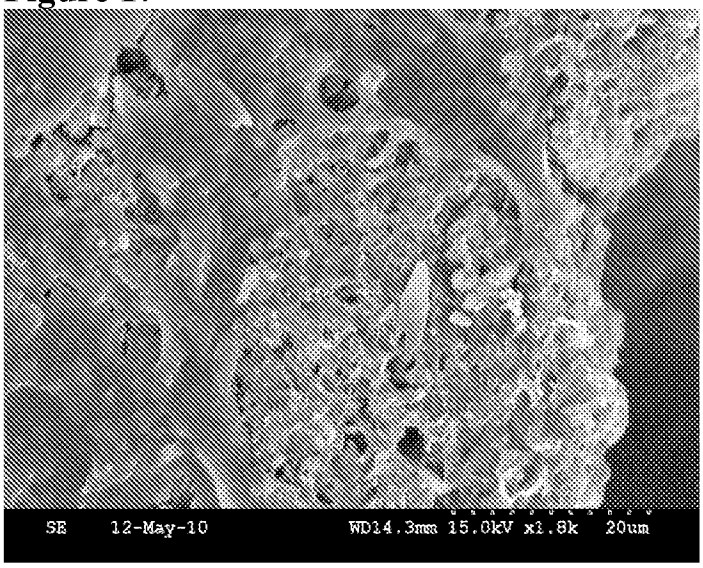


**Figure 18**

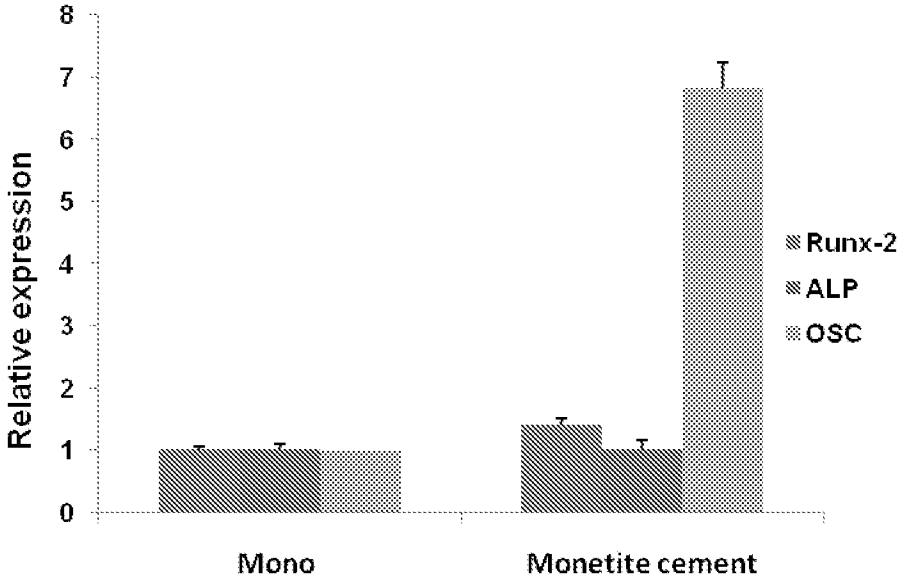



**Figure 19**

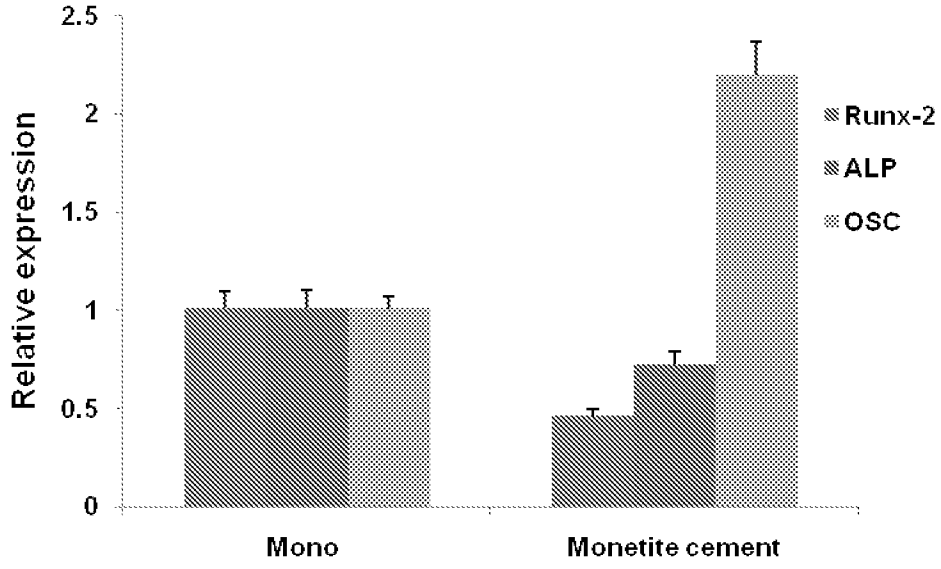


**Figure 20**

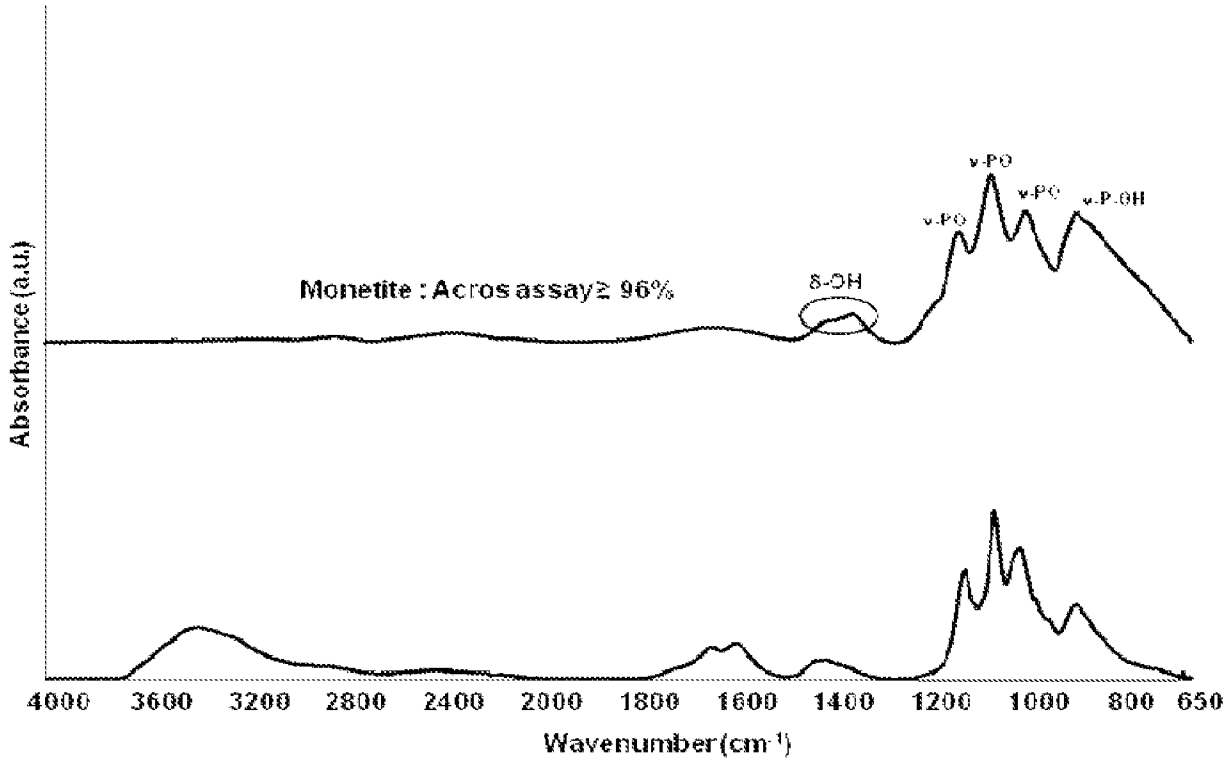



**Figure 21**

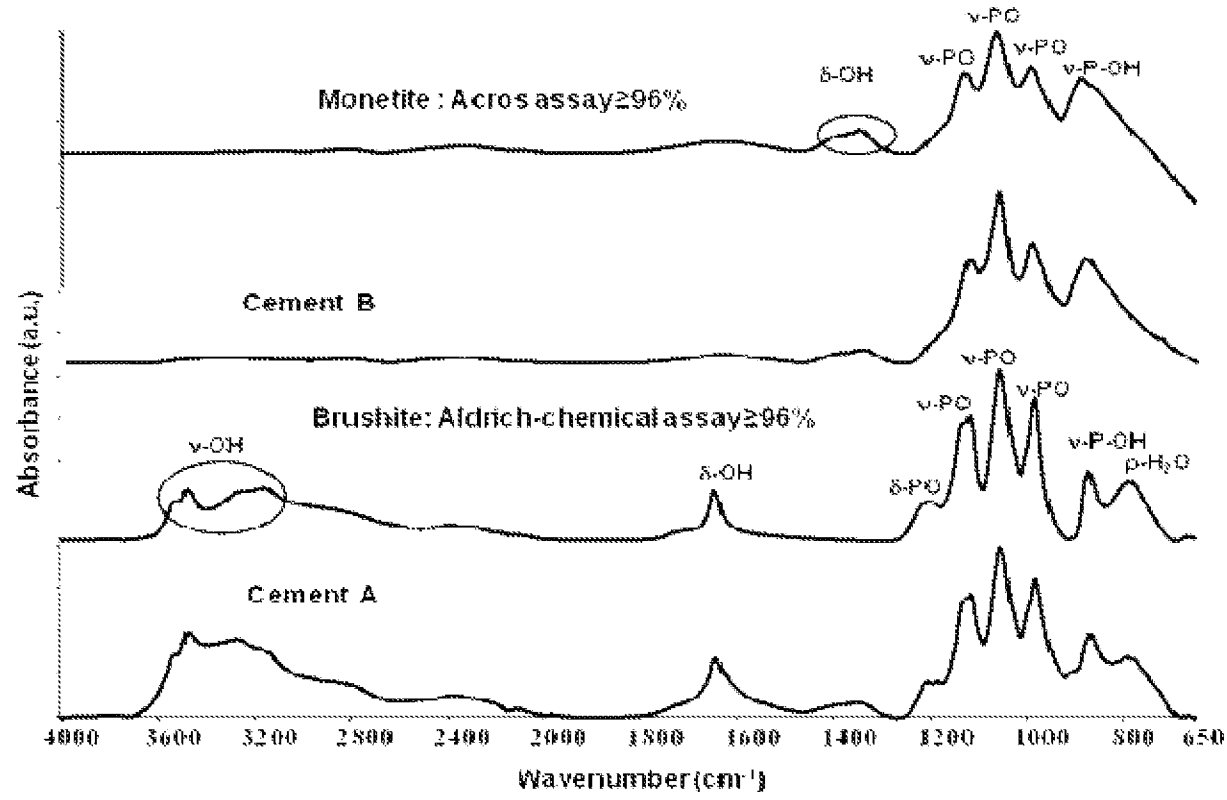


**Figure 22**

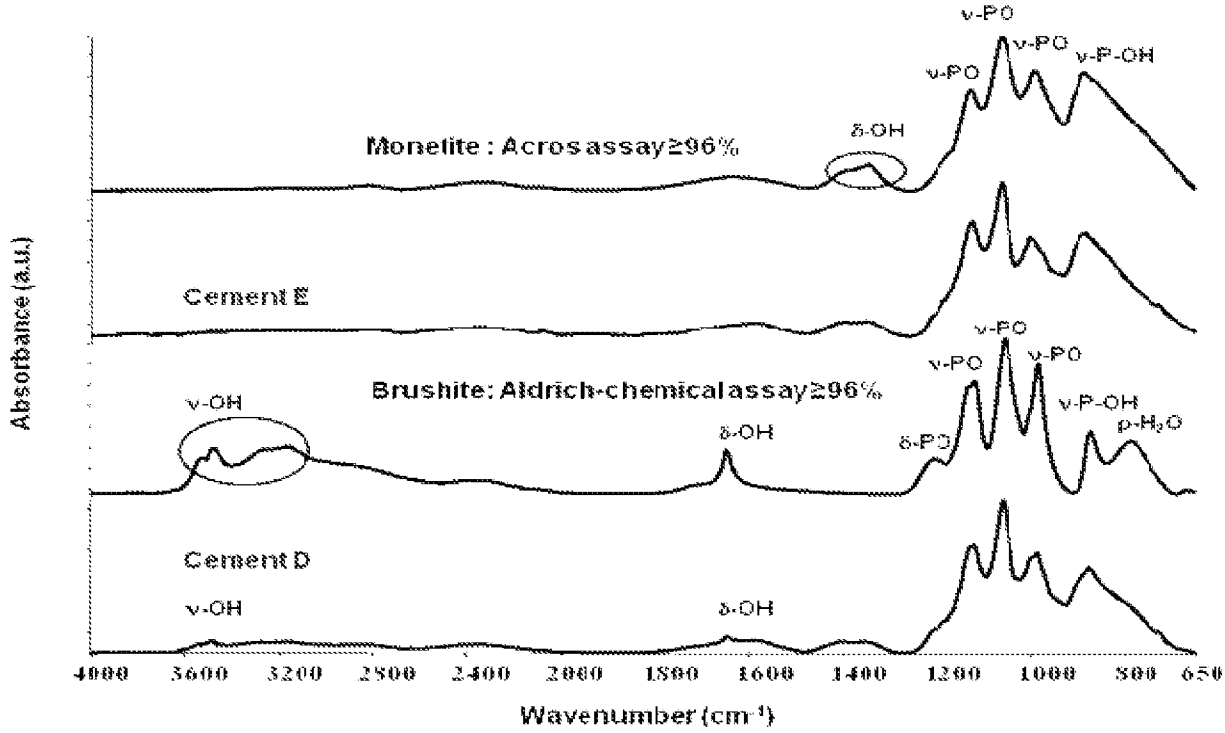
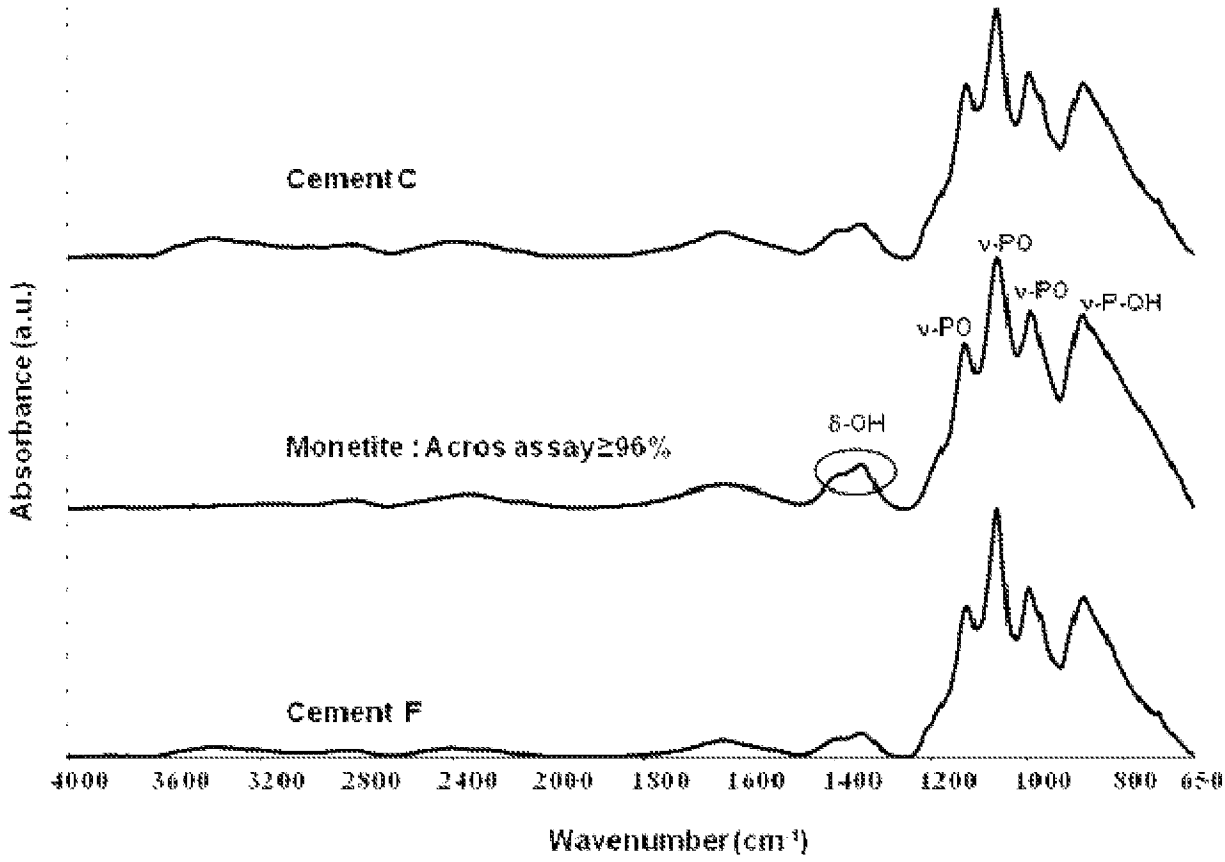



**Figure 23**



# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/050136

A. CLASSIFICATION OF SUBJECT MATTER
INV. C01B25/32 C04B28/34
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C01B C04B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, PAJ, COMPENDEX, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DATABASE WPI<br>Week 198912<br>Thomson Scientific, London, GB;<br>AN 1989-088411<br>XP002676648,<br>& JP 1 037409 A (AOKI H)<br>8 February 1989 (1989-02-08) | 1,22,32 |
| Y | abstract | 2-21,<br>23-31,<br>33-37 |
| | ----- | |
| Y | WO 2004/103419 A1 (ORTUS MEDICAL LTD [GB];<br>BARRALET JAKE [GB]; GBURECK UWE [DE];<br>THULL RO) 2 December 2004 (2004-12-02)<br>page 2, lines 11-19<br>page 5, line 22 - page 6, line 2 | 2-21,<br>23-31,<br>33-37 |
| | ----- | |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 4 June 2012 | 10/07/2012 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Lecerf, Nicolas |

6

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2012/050136

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 38-42
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/** 210

Continuation of Box II.1

Claims Nos.: 38-42

Patents are not to be granted in respect of "methods for treatment of the human or animal body by surgery or therapy and diagnostic methods practised on the human or animal body"; A claim in the form "Use of substance or composition X for the treatment of disease Y..." will be regarded as relating to a method for treatment explicitly excluded from patentability and will therefore not be accepted (Rules 39.1(iii), 67.1(iv) and PCT International Search and Preliminary Examination Guidelines, Chapter 9, Paragraph 9.08-9.10). Claims 38, 39 andclaims 40-42 are regarded as relating to methods of treatment which are expliclitly excluded from patentablility. Claims 38, 39 and claims 40-42 therefore relate to subject matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject matter of these claims (Article 34(4)(a)(i) PCT). For the assessment of the present claims 38, 39 andclaims 40-42 on the question whether they are industrially applicable, no unified criteria exists in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound or of a composition in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 2010/092001 A1 (AZUREBIO S L [ES]; GARCIA DE CASTRO ANDREWS ARCADIO [ES]; GARCIA CARRO) 19 August 2010 (2010-08-19) examples 1-13 | 2-21, 23-31, 33-37 |
| | ----- | |
| A | WO 2010/004066 A1 (HISTOCELL S L [ES]; FONT PEREZ JULIO [ES]; CASTRO FEO MARIA BEGONA [ES) 14 January 2010 (2010-01-14) the whole document | 1-37 |
| A,P | & EP 2 298 696 A1 (HISTOCELL S L [ES]) 23 March 2011 (2011-03-23) paragraph [0008] - paragraph [0011] paragraph [0024] - paragraph [0029] paragraph [0048] - paragraph [0051]; examples 1,2 | 1-37 |
| | ----- | |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2012/050136

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| JP 1037409 A | 08-02-1989 | JP 1037409 A | 08-02-1989 |
| | | JP 1834851 C | 11-04-1994 |
| | | JP 5011048 B | 12-02-1993 |
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| | | EP 1633409 A1 | 15-03-2006 |
| | | US 2006213398 A1 | 28-09-2006 |
| | | WO 2004103419 A1 | 02-12-2004 |
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