Biorepair® PLUS

THE ENAMEL REPAIRER

LEBSC
Laboratorio di Strutturistica Chimica Ambientale e Biologica
Laboratory of Enviromental and Biological Structural Chemestry

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

I am pleased to present BioRepair® Plus to you.

BioRepair® Plus, developed by Coswell Research Laboratories in collaboration with the LEBSC (Laboratory of Environmental and Biological Structural Chemistry) at the University of Bologna, Italy, is the first toothpaste based on bioactive microparticles which, thanks to their composition, are able to penetrate into the microscopic defects on the enamel and dentine surfaces, performing an effective remineralising and reparative treatment.

The LEBSC has been operating for thirty years and uses the most advanced techniques to study the chemical and biological aspects of mineralization processes of calcified tissues and, in particular, bone tissue.

Enclosed is an in-depth scientific study which supports the effectiveness of BioRepair® and its MICROREPAIR® action

Used daily, BioRepair® will repair tooth enamel and will make your teeth more healthy – definitely something to smile about!

Thank you for your time.
Yours faithfully,

Prof. Norberto Roveri
Full Professor of General and Inorganic Chemestry
"G. Ciamician" Department of Chemistry,
University of Bologna

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Introduction

Like vertebrate bone tissues teeth are a composite natural material, the main component of which is an inorganic carbonated hydroxyapatite phase, representing 65-70% of dentine by weight and over 90% of enamel by weight.
Dental enamel forms the thin outer coating of our teeth and is considered to be the hardest and strongest of all biogenic materials (figure 1).

Tooth enamel is composed mainly of large, prismatic, highly crystalline hydroxyapatite microparticles , together with a very low protein component (figs. 2a and 2b).

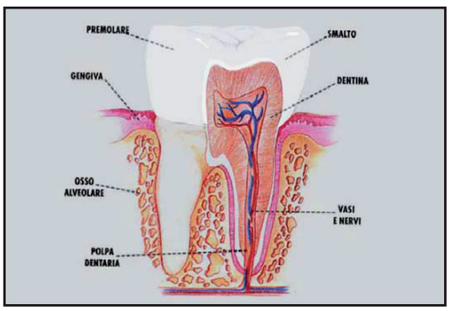


Figure 1: Tooth structure

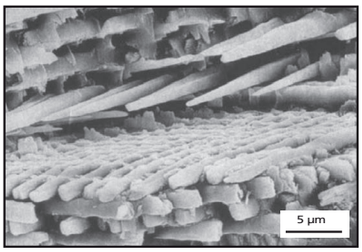


Figure 2a: Enamel structure viewed with Scanning electron microscopy (SEM) (Scale bar=5µm). Reproduced with permission from H. Lowestan and S. Weiner, "On Biomineralization", Oxford University Press 1989.

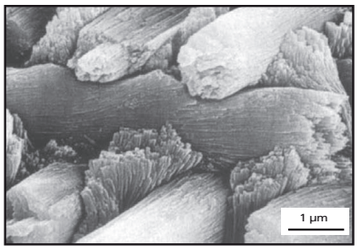


Figure 2b: SEM image: layers of rod-like hydroxyapatite microparticles that form enamel (Scale bar=1µm). Reproduced with permission from H. Lowestan and S. Weiner, "On Biomineralization", Oxford University Press 1989.

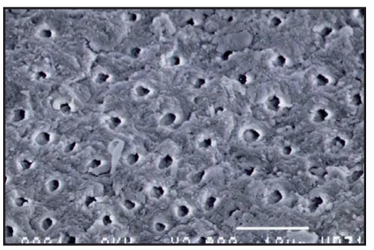


Figure 3: Dentine structure viewed using Scanning electron microscopy (SEM). The dentinal tubules can be seen clearly, occupied by the odontoblast processes and intratubular fluid implicated.

In adults, enamel does not contain any cells and is therefore not capable of self regeneration. Any damage is irreversible, as there is no biological process capable of repairing damaged enamel. In a similar way, dentine (figure 3) exposed to the oral environment cannot be regenerated, as new dentine is deposited on the interior surfaces of the crown close to the pulp, and not on the outside. For this reason, any reparative action must be provided by materials or substances that are extraneous to dental tissue metabolism. These substances are either synthetic or are precipitated from saliva.

MICROREPAIR®

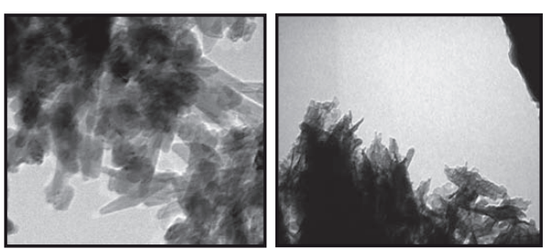
The hydroxyapatite microparticles (MICROREPAIR®) present in BioRepair® Plus are completely identical to the mineral that forms dentine and enamel. It is precisely from this similarity that the synthetic microparticles derive their capacity to reconstruct dentine and enamel.

BioRepair® Microparticles

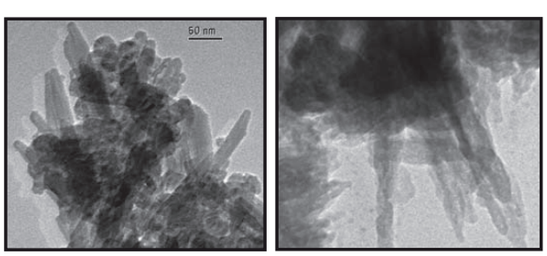
$$Ca_{(10-x)}Zn_x(PO_4)_{(6-y)}(CO_3)_y(OH)_2$$
$$(Y=4\text{-}8\ \%\ CO_3^{2-}\ \text{substituted at}\ PO_4^{3-})$$
$$(X=1\ \%\ Zn^{2+}\ \text{substituted at}\ Ca^{2+})$$

Figure 4: Chemical formula for dentine and enamel remineralising hydroxyapatite microparticles (Microrepair®). Calcium phosphate and carbonate are present in very similar relationships to those occurring naturally. It is the zinc, with its antiseptic properties, that is responsible for the plaque-preventing action.

The substance used in BioRepair® Plus is technologically innovative, in that being in the form of microparticles it has enhanced chemical reactivity. Microparticles carry out their dentine remineralising action by releasing their constituent calcium and phosphorous in situ. In the case of the enamel, the microparticles action takes place via their ability to bond to natural tissues, thus filling microgaps in the enamel.



Figures 5 and 6: Microscopic Microrepair® microparticles aggregates viewed using TEM (Transmission electron microscopy) (Scale bar=100 µm)



Figures 7 and 8: Microscopic Microrepair® microparticles aggregates viewed using TEM (Transmission electron microscopy (Scale bar=50 and 20 µm respectively).

Introduction

Action mechanism

The enhanced reactivity of MICROREPAIR® microparticles is due primarily to the bio-mimetic action of these microparticles, which have the distinguishing feature of possessing a very similar chemical composition to that of enamel and dentine. The X-ray diffraction spectrum of MICROREPAIR® microparticles (figure 9) shows how the degree of crystallinity of the microparticles is half way between that of enamel and dentine.

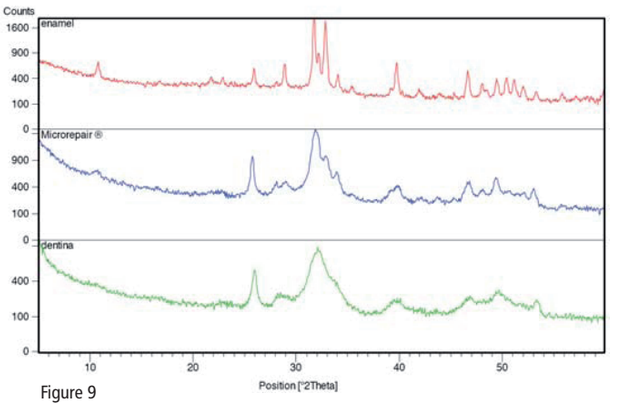


Figure 9: X-ray diffraction diagrams of enamel (red line), MICROREPAIR® microparticles (blue line) and dentine (green line).

Figure 9

The ability to remineralise hard tissues has an impact on several disorders that affect the hard tissue of teeth:

1. Prevention of cavities by **remineralisation** of original lesions;
2. **Desensitising** effect on dentine by covering up dentinal tubules;
3. **Tartar** and **plaque** prevention thanks to antibacterial action of Zn^{2+}, with recognised antiseptic properties

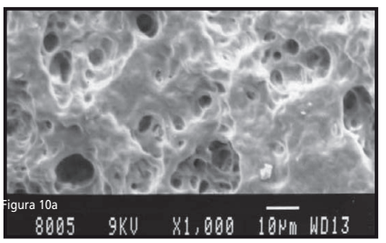


Figure 10a

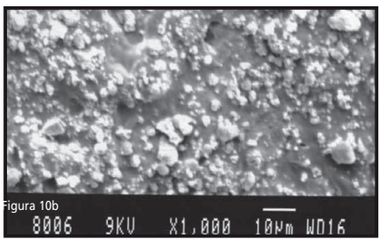


Figure 10b

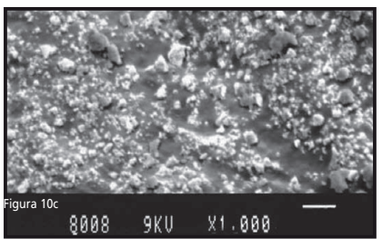


Figure 10c

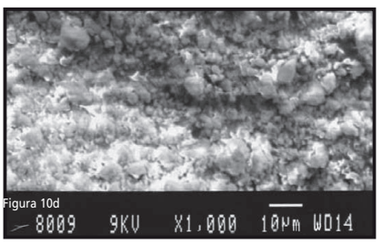


Figure 10d

Figure 10: the SEM images show the surface of the dentine before treatment (a) and after treatment with MICROREPAIR®: 1 minute (b), 10 minutes (c) and one hour (d). They clearly show the progressive growth of the apatite microparticles until they completely hide the dentine channels.

Figures 10 (a, b, c and d) show the progressive action of MICROREPAIR® microparticles, as they gradually bond securely to the surface of the dentine, blocking the tubules and thus performing their effective, long-lasting desensitising effect on the dentine.

The phenomenon can be seen at work a few minutes after the BioRepair® Plus microparticles have been applied, which means that the microparticles remineralising and desensitising action begins after only a few applications of BioRepair® Plus toothpaste.

A similar mechanism can be seen on the surface of the enamel where there are microscopic defects or simple surface irregularities, as the BioRepair® Plus microparticles are laid down and begin their recrystallisation process.

The Zinc release mechanism is extremely innovative, as it takes place via slow dissolution of the apatite, which then releases its various components locally (Zn^{2+}, Ca^{2+}, CO_3^{2-}, PO_4^{3-}). The Zinc, in its ionic form as a bivalent action, then performs its antiseptic function inside the mouth.

The Zinc is released at an optimum concentration, therefore carrying out its antiseptic action in the mouth.

4. Finally, apatite has other secondary effects, including the absorption of sulphate compounds such as H_2S, which is responsible for halitosis. Daily use of BioRepair® Plus is therefore also indicated for control of this condition: thanks to the absorbent properties of BioRepair® Plus microparticles , daily use of Microrepair® is effective in fighting halitosis problems.

Repairing Enamel

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Teeth

Enamel is the hardest material in vertebrates and is the most highly mineralized skeletal tissue present in the body [1]. Mature enamel is composed of carbonated hydroxyapatite (CHA) (95-97%wt) and less than 1% wt of organic material. Unlike other biomineralized tissues, such as bone and dentine, mature enamel has no cells and therefore cannot be biologically remodelled. Consequently, enamel regeneration cannot take place in vivo. Affording this situation is therefore an attractive target for future innovative biomimetic and therapeutic approaches for the health protecting possibilities involved.

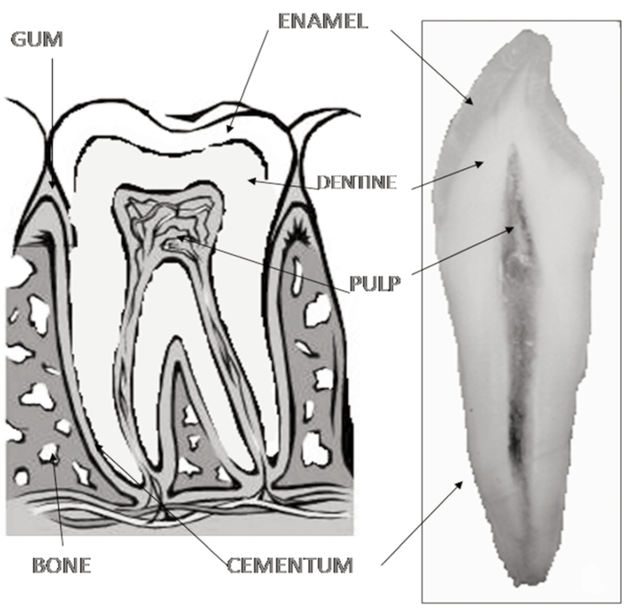


Fig.1 Tooth anatomic draft: enamel (E), dentine (D), pulp (P) and cementum (C).

The mammalian tooth is made up of four distinct structures: enamel, dentine, pulp, and cementum.

Enamel (E) is the outermost layer on the crown of the tooth and situated above the dentine (D). The pulp (P) contains nerves and blood vessels, while the cementum (C) is the outermost layer of the mineralized tissue surrounding the root of the tooth allowing the tooth, keeping it anchored to the jawbone through the periodontal ligament (Fig. (1)) [2].

The pulp contains nerves, blood vessels, fibroblasts and lymphocytes, while the tooth mineralized area concerns enamel, dentine and cementum. Enamel makes up the uppermost 1-2 mm of the tooth crown and contains a high mineral content, giving it a high modulus, but also making it susceptible to cracking. Dentine lies below the enamel and is tougher, forming the bulk of the tooth and absorbing stresses from enamel, preventing its fracture [3]. The cementum is the mineralized layer that surrounds the root of the tooth covering the dentine layer and anchoring the tooth to the alveolar bone (jawbone) through the periodontal ligament. The primary function of the teeth is for mastication of food and it is very important to maintain them healthy keeping their mechanical properties long life lasting in a bacteria-filled environment.

Enamel prismatic HA microparticles consist of a weaving of prisms ranging from 3 to 5 μm in diameter. A single prism reveals a highly organized array of fastened needle like HA crystallites (approximately 30 nm thick, 60

nm wide, and several millimetres long) (Fig. (2A)). They are preferentially aligned along the HA crystallographic c-axis organized in interweaving bundles of aligned crystallites that are "woven" into intricate architectures approximately 3-5 μm in diameter (Fig. (2B)) [4].

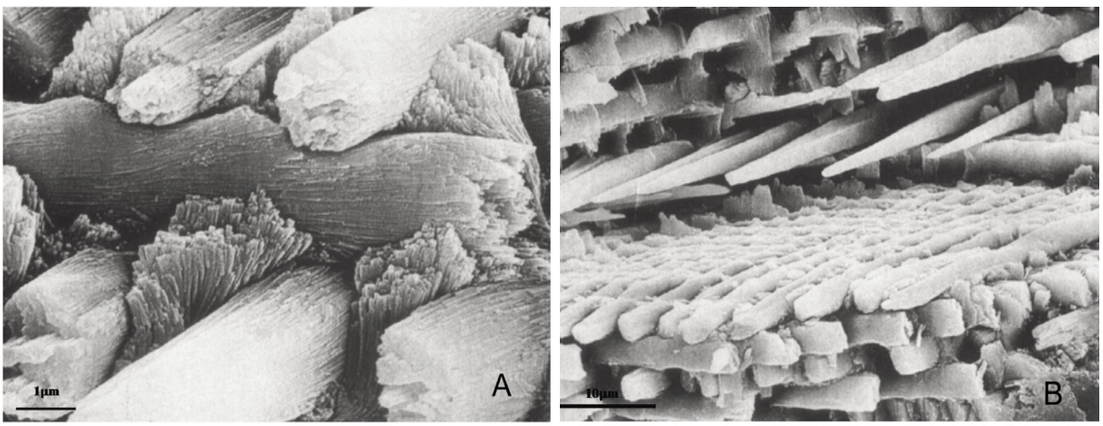


Fig.2 Scanning Electro Microscopy enamel images of highly organized array of fastened needle like HA crystallites (A) organized in interweaving bundles into intricate architectures (B).

The composition and morphology of dentine resembles that of bone and is characterized by exhibition of numerous tubules containing nervous tails (Fig. (3)).

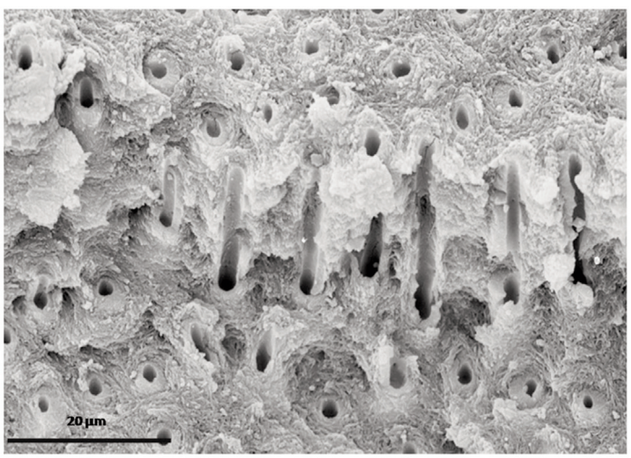


Fig.3 Scanning Electro Microscopy image dentine showing tubules in a bone like morphology.

Enamel and dentine are tough, crack-tolerant, and abrasion-resistant tissues for their unique architectures and mineral compositions. Because of the high mineral content and minimal organic one, enamel is brittle. It is interesting to note that the architecture of the enamel crystallites can deflect a propagating crack, preventing this from reaching the dentine-enamel junction (DEJ), a gradual transition from dentine to enamel and has been shown to resist to the tissues de-lamination despite their different composition [5].

Teeth hearth prevention

Dental erosion is the chemical wear of the dental hard tissue without the involvement of bacteria [6]. Its clinical relevance is becoming wider and wider [7-11], and it is considered one of the main tooth pathologies able to cause patient discomfort, after periodontal diseases and caries.

Erosion aetiology appears related to the enormous increase in consumption of soft drinks, fruit juices and sport drinks consumption [12]. However, other acid sources, such as drugs containing syrups, analgesics and vitamin C intake, and environmental acid exposure in working condition are claimed to be related to enamel erosion development [13-17].

The mechanisms involved in the damage of dental hard tissue are related to the acid attacks on the outer few micrometers of the enamel, with the consequent demineralization and dissolution of the minerals [18-24].

Dentine hypersensitivity, defined from Holland et al. [25] as "the short sharp pain arising from exposed dentine in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other form of defect, pathology or disease" [26], has been explained in term of hydrodynamic mechanism [27]. Essentially, if dentine is exposed and the dentine tubules are open, the fluid movement provokes stimulation [28]. Even if much remains unknown or unproven about the aetiology of dentine hypersensitivity, according to many authors it can be considered a tooth wear phenomenon [29]. The smear layer, which is an artificial surface about one micron thick, consisting of collagen and hydroxyapatite from the native dentine, is formed when dentine is abraded [30]. It covers the underlying dentine occluding the tubules, but can be removed by attrition, acid erosion and tooth brushing with toothpaste [31,32]. Therefore a considerable overlap exists between the aetiology of dental erosion and that of dentinal hypersensitivity. To prevent dental erosion progression, the reduction or elimination of exposure to acidic soft drinks and juices can be recommended to patients. Frequent application of a high concentration of topical fluoride may be of some benefit in preventing further demineralization and increasing the abrasion resistance of erosion lesions [33]. In–vitro studies have shown that inhibition of dissolution of synthetic carbonated-hydroxyapatite is a logarithmic function of the fluoride concentration in solution [34].

The demineralised area and micrometric sized scratches normally occur on enamel surface as a consequence of micro wear and acid attack [35] and cannot be repaired biologically, nor prosthetically. In fact, unlike in bone, when apatitic microparticles are dissolved or abraded, they cannot be spontaneously re-deposited in enamel and dentine because enamel contains no cells able to secrete extra-cellular matrix. The regeneration and dentine apposition occurs only through the pulp tissue. Therefore, both enamel and dentine can be reconstructed only by the application of all plastic materials which provide a sort of prosthetic restoration.

Most of the products and devices commonly used to counter enamel and dentine erosion such as fluoride [36-39], work by reducing apatite dissolution and increasing surface micro hardness [40- 42], but are unable to reconstruct the lost mineral.

Hydroxyapatite is the main constituent of the dental tissues representing in enamel and dentine the 95 wt % and 75 wt % respectively and, like in bone, it is the main responsible for the mechanical behaviour of the dental tissues.

Hydroxyapatite has been widely experimented as a biomaterial, thanks to its biocompatibility and osteoconductivity. It represents an elective material covering a wide range of biomedical applications for bone substitution and bone-prosthesis interface [43]. Poorly crystalline HA microparticles, in addition to the excellent biological properties of HA (non-toxicity and lack of inflammatory and immunizing responses) can be biodegradable in physiological conditions. This property can be modulated by modifying its degree of crystallinity, which recently can be achieved by implementing innovative synthesis able to control crystal growth at nano size level. In the last decade, advanced technology has been utilized to synthesize a new generation of biomimetic apatitic all plastic materials which can optimize the interaction with biological entities thanks to their strong surface bioactivity [44]. The aim of this paper is to review the main patents concerning oral care fluoridated products and the recent patents in which a daily use of synthetic apatite instead of fluoride for health care is recommended.

Remineralization by fluoride

In vitro, fluoride (0.02-0.10 mg/L) addition to a supersaturated solution of calcium phosphate induces the crystallization of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ which is the mineral phase of bone and teeth. Increasing fluoride concentration fluoroapatite $Ca_{10}(PO_4)_6F_2$ is formed and appears in more ordered and bigger apatite microparticles which are less acid soluble [45-47].

In the body, in vivo fluoride is mainly associated with calcified tissue, bone and teeth due to its high affinity to calcium. Fluoride modifies the bone mineral phase by replacing the hydroxyl groups in hydroxyapatite phase producing its partial conversion into fluoroapatite. The more electrostatic stability and crystallinity of fluoride substituted hydroxyapatite increases the bone density and hardness reducing the mechanical

strength [48,49]. Fluoride in high doses was found to be mutagenic in osteoblasts and inhibitory of osteoclasts [50-52,48].

In spite of the observed negative physicochemical effects on the bone, fluoride exhibits both a cariostatic effect on children and adults erupted teeth and a pre-eruptive effect through increasing fluoridation of the developing enamel [53,54]. Fluoridated enamel is less acid soluble [55]. The reduction of caries in both deciduous and permanent teeth was more marked where the children were earlier exposed to fluoridated tip water [53,56]. The fluoride cariostatic effect on erupted teeth can be ascribed to an inhibition of the demineralization of sound enamel due to ingested acid foods and drinks and cariogenic bacteria in the dental plaque. Sound enamel results more acid resistant containing more fluoride [57,58]. However, caries is not a fluoride deficiency disease and no specific fluoride deficiency syndrome has been found.

Adsorbed fluoride is rapidly distributed by circulation to the intracellular and extracellular fluid, is retained only in calcified tissues and the fluoride plasma concentration is dependent on the ingested fluoride dose. Fluoride concentrations in oral environment and glandular saliva closely follow the plasma concentration, but at a lower level about two-thirds of the plasma level [59]. This finding suggests that the fluoride concentration in saliva and dental plaque is strongly dependent on topical fluoride application by dental care products [57,60-74]. Children with no caries experience were found to have higher salivary fluoride concentrations than children highly affected by caries [75].

Contrary to skeletal bone and dentine which accumulate fluoride in proportion to the fluoride adsorbed during life, enamel reflects the biologically available fluoride during teeth formation.

Enamel maturation of deciduous teeth is completed between the age of 2 to 12 months while in permanent teeth enamel maturation is completed at the age of 7-8 years except for the third molars whose maturation continues until the age of 12-16 years. Post-eruptive fluoride uptake of enamel is expressed only in the outer layer and depends on fluoride in saliva, food, drinks, dental plaque and prevalently in dental products. In order to prevent enamel demineralization by acid food and drinks and cariogenic bacteria in the dental plaque, many dental products as toothpaste, rinses and gels containing fluoride have been recommended by medical societies in many countries for caries prevention. The use of fluoridated oral care products are recommended especially in countries where the fluoride concentration from drinking water is low even if there are considerable differences regarding the starting time (birth-6 months of age) and amounts related to age. However fluoride from toothpaste swallowed by a four year old child was found to contribute up to one third to one half of total daily fluoride intakes of 3.6 and 2.3 mg respectively [76]. In the European communities about 90% of all toothpastes are fluoridated in the range from 1000 mg/kg to 1500 mg/kg with a maximum level of 1500 mg/kg. The Scientific committee on Cosmetic products and non-Food Products Intended for Consumers (SCCNFP, 2003) states that the amount of toothpaste applied to the toothbrush for a child below the age of 6 years can vary between 0.05 and 0.8 g. The recommended "pea size" amount is considered to be 0.25 g. According SCCNFP simulation model the fluoride ingestion from such toothpastes could amount up to 50% of the adequate fluoride intake of children at that age. In the model calculation for 3-5 year old children in the USA the fluoride intake from ingested toothpaste was estimated to be 30-60% of the dietary [77].

Fluoride tolerable intake

Fluoride is not essential for human growth and development and its content in the body is not under physiological control. Adsorbed fluoride is rapidly distribute by circulation to the intracellular and extra cellular fluid, but is retained only in calcified tissues. Plasma fluoride occurs in both ionic and non-ionic forms which consists mostly of fat-soluble fluorine compounds. In adults, adsorbed fluoride is only partially less than 50% retained in skeleton and the remainder excreted prevalently via the kidney. On the contrary, in infants fluoride retention in bone can be as high as 90% and appears also incorporated into dental enamel during teeth formation. Excessive intake of fluoride during enamel maturation from birth to eight years of age , when enamel formation is complete, can lead to reduced mineral phase content of enamel and to dental fluoro-

sis of deciduous, but prevalently of permanent teeth. Dental fluorosis has been usually associated with increased resistance to caries, but it is increasing the consideration of appreciable fluorosis as an adverse effect [78]. While mild dental fluorosis is not readily apparent, moderate dental fluorosis can be easily appreciated and is characterized by white spots and opaque striations staining and minute pitting of teeth (Fig. (4)) [79]. Fluorides in toothpaste, whilst well known for their anti-caries benefits, are toxic if ingested at high levels, in particular in children because of an adverse dose to weight ratio [80,81].

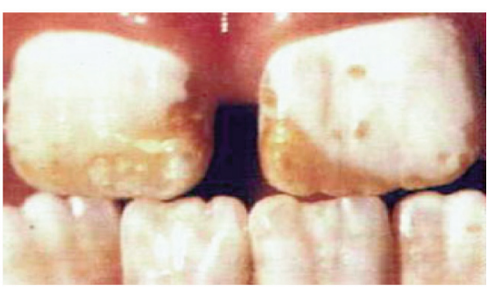
The International Standards Organization Toothpaste minimizes this safety matter by fixing the maximum fluoride dose allowed in oral care products. Furthermore, children products contain a lower fluoride concentration, and therefore a lower dose, and/or manufacturers recommend a small amount of adult toothpaste to be placed on the brush. The recently produced toothpastes containing a very high fluoride concentration exceeding the International Standards Organization limits are exclusively prescribed by professionals and are not recommended for children [82]. The European Food Safety Authority (EFSA) Scientific Panel considers that the maximum fluoride intake is 0.1 mg fluoride/kg/day in children aged 1-8 years which is equivalent to 1.5 and 2.5 mg fluoride per day in children aged 1-3 years and 4-8 year respectively.

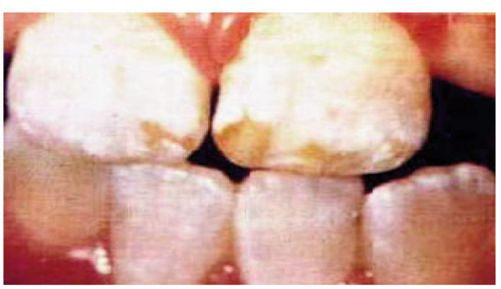
Fluoride accumulation in skeleton changes bone mechanical behaviour reducing bone strength and increasing its density and stiffness, causing skeletal deformities and risk of fractures. Therapeutic studies with fluoride in postmenopausal osteoporosis suggest an increasing risk for skeletal fractures at or above fluoride intakes of 0.6 mg/kg body weight per day. Fluoride increases with age in bone, more rapidly in women than in men and preferably in cancellous bone [83,84].

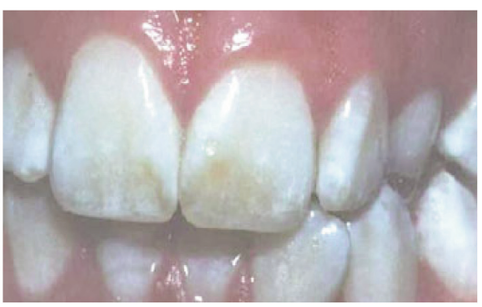
Fluoride is not irreversibly bound to bone and can be released during remodelling of bone [85].

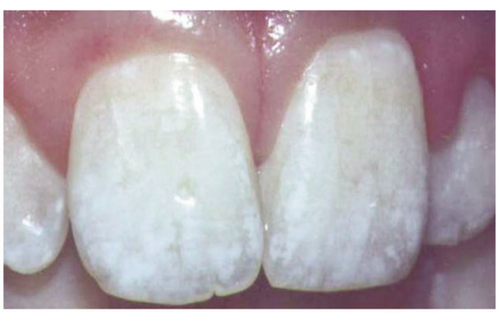
Excluding the fluoride exposure via inhalation and the skin which in normal circumstances is really negligible, fluoride intake is due to oral ingestion by drinking water, beverages, foodstuffs, including fluoridated salt, dental health products and fluoride tablets for caries prevention.

Fluoride concentration in drinking water (0.3-1.5 mg/L) differs according to the countries' natural circumstances and to water fluoridation (U.K., Ireland, Spain and Switzerland). This has been recently reduced or terminated. In a 2004 Water Quality Report for a certain public water supply, the following Public Notice was issued regarding Fluoride: "This is an alert about your drinking water and a cosmetic dental problem that might affect children under nine years of age. At low levels, fluoride can help prevent cavities, but children drinking water containing more than 2 mg/L of fluoride may develop cosmetic discolouration of their teeth (dental fluorosis). Dental fluorosis, in its moderate or severe forms, may result in a brown staining and/or pit-









ting of the permanent teeth (Fig.(4)).

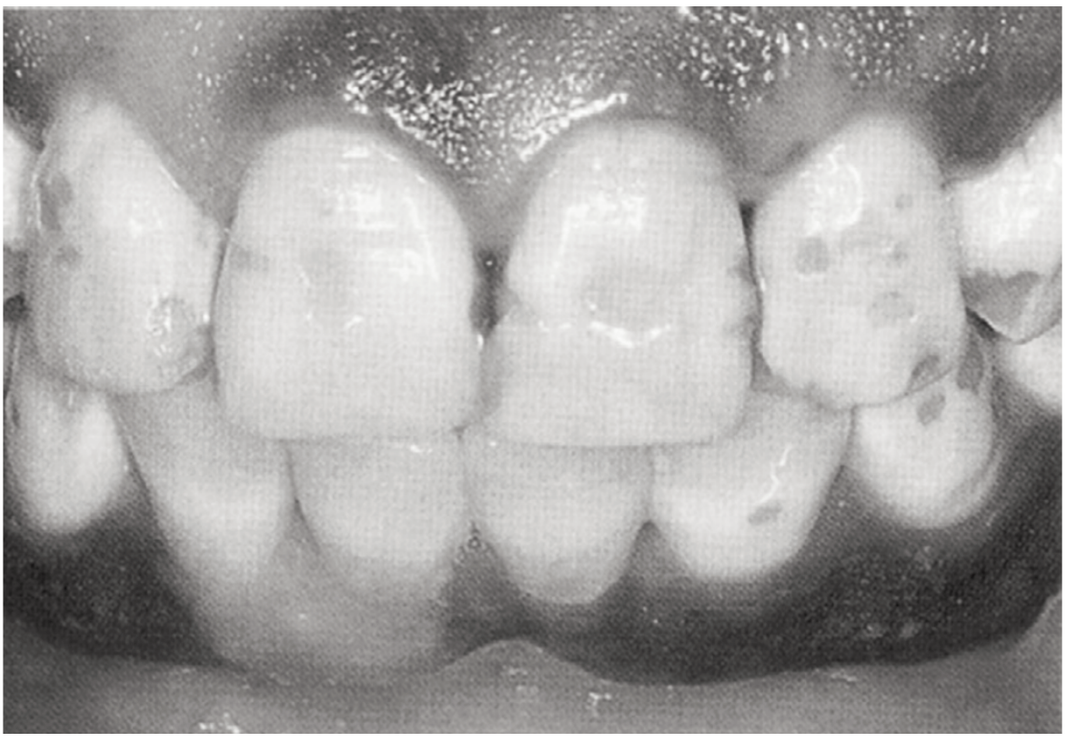


Fig.4 Fotografic images of teeth affected by dental fluorosis.

This problem occurs only in developing teeth, before they erupt from the gums. Children under nine should be provided with alternative sources of drinking water or water that has been treated to remove fluoride to avoid the possibility of staining and pitting of their permanent teeth. You may also want to contact your dentist about proper use by young children of fluoride-containing products. Older children and adults may safely drink the water. Drinking water containing more than 4 mg/L of fluoride (the U.S. Environmental Protection Agency's drinking water standard) can increase your risk of developing bone disease. Your drinking water does not contain more than 4 mg/L of fluoride, but we're required to notify you when we discover that the fluoride levels in your water exceed 2 mg/L because of this cosmetic dental problem. Some home water treatment units are also available to remove fluoride from your drinking water" [79].

Vegetables and fruit containing from 0.02 to 0.20 mg/kg fresh weight, milk and milk products 0.05-0.15 mg/kg, meat and meat products 0.15-0.29 mg/kg, eggs 0.18 mg/kg, fish 0.48-1.91 mg/kg.

Exceptions are tea which can contain considerable amounts of fluoride (0.34-5.2 mg/L), and some brands of instant teas were reported to contain significant amounts of fluoride even 6.5 mg/L .On the contrary, in human milk the fluoride concentration is about 0.2 mg/L.

Children aged 1-8 years get fluoride intakes from food and water usually far below the UL. The clear increase of mild dental fluorosis occurred in some countries has been ascribed to the inappropriate use of fluoridated dental care products in particular fluoridated toothpastes. Dental products like toothpaste, rinses and gels containing fluoride can increase the total intake of fluoride especially when inappropriately used [86]. This happens especially in children younger than 7 years ingesting high amounts of toothpaste [87-90]. In the model calculation for 3-5 year old children in the USA the fluoride intake from ingested toothpaste is estimated to be 30-60% of the dietary CTE.

For these reasons, in the European Communities all toothpastes are fluoridated with a maximum level of 1500 mg/kg. The need to discover and develop an alternative to fluoride for teeth health prevention care is clearly opportune.

Biogenic hydroxyapatite

Vertebrate bones and teeth are biological hybrid materials where a calcium phosphate, in the form of hydroxyapatite (HA), represents the inorganic component intimately inter grown with the organic matter prevalently constituted of proteins and polysaccharides [91,92]. Biological HA is not stoichiometric according to the ideal formula $Ca_{10}(PO_4)_6(OH)_2$, but at low extent Ca^{2+} is replaced by other ions like Zn^{2+}, Sr^{2+}, Na^{+}, K^{+}, Mg^{2+} while PO_4^{3-} and OH^{-} can be partially substituted by other anions like CO_3^{2-}, HPO_4^{2-}, $P_2O_7^{4-}$, SiO^{4-}.

The bone mineral phase is more correctly called carbonate hydroxyapatite. Carbonate is the prevalent foreign anion and represents about 4-8 wt % [93,94]. The substitution of CO_3^{2-} groups into the PO_4^{3-} sites (type B carbonate apatite) is prevalent in young humans, while the carbonate replacement to OH^{-} groups (type A carbonate apatite) increases with the age of the individual [95].

The bone carbonate hydroxyapatite microparticles, which can represent a typical example of an "organic matrix-mediated" biogenic material, have a blade shape of approximately 25 nm width, 2 - 5 nm thickness and about 60 nm length. Biogenic hydroxyapatite microparticles exhibit nonstoichiometric composition, structured carbonate ions in the crystal lattice, low degree of crystallinity, plate acicular morphology and a nano size which give a large surface area of about 120 m^2/g (Fig. (5)) [92,96,97].

Vertebrate bone can be considered a "living biomaterial" since it contains inside a network of different cells under permanent activity, living within the mineralized structure, and are interconnected through pores and channels. A dynamic process of bone formation and destruction accounts for its growth during the body development and regeneration after fractures.

Dentine, resides within the central region of the tooth and is similar to bone in composition and structure [98-107]. Enamel, the tooth external surface coating, has a much larger inorganic content than bone and

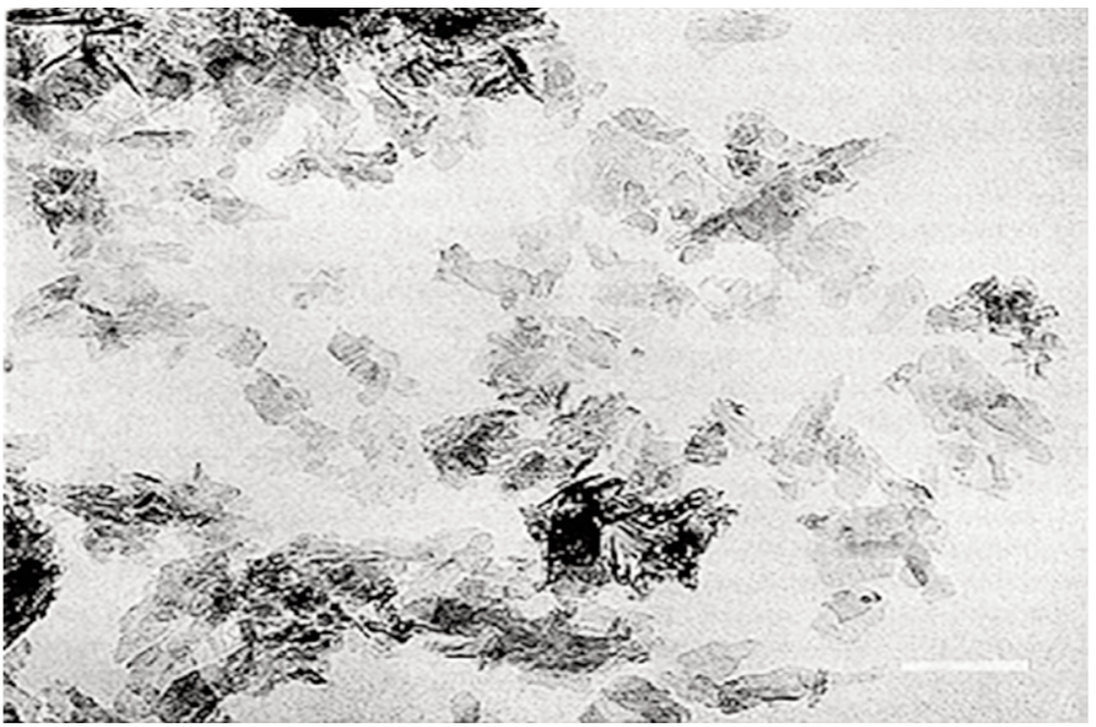


Fig.5 Transmission Electron Microscopy image of deproteinated bone hydroxyapatite microparticles.

dentine, close to 95% wt, which is mainly constituted of long thin ribbon-like prismatic microparticles of hydroxyapatite, that exhibit a higher degree of crystallinity and a lower carbonate content than bone and dentine apatite microparticles. Amelogenins, present in relatively large amount in the early stages of enamel formation, are enzymatically degraded and removed up to 5% wt as the hydroxyapatite microparticles grow [108]. Adult dental enamel, considered the most resistant and tough material in the biological world,

does not contain cells and therefore cannot be regenerate itself. There is no biological process that can repair degraded or damaged enamel, evidencing the need for synthetic enamel biocompatible materials able to repair teeth decay [109-111].

Biomimetic synthetic hydroxyapatite

Biomimetism of synthetic materials for biomedical applications can be carried out at different levels in view of composition, structure, morphology, bulk and surface chemical-physical properties.

Biomaterials can be turned biomimetic imprinting all these characteristics in order not only to optimize their interaction with biological tissues, but even to mimic biogenic materials in their functionalities. Chemists, biologists, physicists and engineers interested in material science are amazed by the high degree of sophistication, miniaturization, hierarchical organization, hybridising, reliability, efficiency, resistance and adaptability characterizing natural materials.

These properties which biogenic materials have achieved through specific building principles selected by evolution, can be only partially obtained in manmade materials by present synthetic processes. For this reason Nature is a school for material science. Biomimetism and bioinspiration represent important tools for the design and the synthesis of innovative materials and devices [112- 116].

The highly elaborated performances of biologically occurring materials are the results of an evoluted convergence on limited constituents, which occur at a precise moment, and are available at that time. Nature produces soft and hard materials exhibiting remarkable functional properties by controlling the hierarchical assembly of simple molecular building blocks from the nano to the macroscale [117]. Biomineral morphogenesis is related to specific strategies for the long-range chemical construction of well organized architectures from preformed nano or micro crystalline inorganic building blocks. In fact, many biologic complex structures are obtained by promoting specific links induced by the conformation variability at the nanometre scale of biological macromolecules. Biosystems reveal a high level of integration of three fundamental aspects: the nano-micro "spatial confinement" of biochemical reactions, the inorganic and organic "hybridization" compounds and the "hierarchy" from nano to macro scale, in order to produce a biomaterial able to exhibit the appropriate chemical-physical properties at any different scale level [118-121]. Biogenic materials are nucleated in defined nano-micro dimensioned sites inside the biological environments in which chemistry can be spatially controlled. The spatial delimitation is essential to biological mechanisms to control size, shape and structural organization of biomaterials.

With the development of nanotechnology, this strategy employing natural material genesis, has attracted a lot of attention in designing bioinspired materials such as polymeric micelles, microparticles, dendrimers and microparticles synthesized in nanoscale dimensions [122-127].

Since the last 30 years calcium phosphate ceramics have been, and still are nowadays, very popular implant materials for diverse clinical applications. Porous HA, simulating spongy bone morphology has been prepared using various technologies to control pore dimension, shape, distribution and interconnections. HA ceramics processed by high-temperature treatment [128] present a significant reduction of bioreactivity and growth kinetics of new bone due to the not resorbability. New synthetic methods at lower temperatures have been developed, allowing one to obtain porous bioceramics with a low degree of crystallinity. Colloidal processing [129], starch consolidation [130], gel casting and foam out [131] have yielded excellent results, producing bioceramics with a bimodal distribution of the pore size that can be modified as a function of the sintering conditions.

Different types of CaP-ceramics are available, though they can be classified as either hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), biphasic calcium phosphate (BCP), amorphous calcium phosphate (ACP), carbonated apatite (CA) or calcium deficient HA (CDHA) [132]. The use of these materials for tissue engineering purposes is still explored. Most researchers are aware that the low resorbability of sintered CaP-ceramics, and in particular the incomplete resorbability of ceramic HA, appears useful when biomaterial has to be implanted with a defined 3D form. The use of highly porous implants induces bone formation inside the

implant and increases degradation, but the complete resorption in most cases is very difficult due to the crystalline architecture.

Porous coralline HA can be synthesized by a hydrothermal method for HA formation directly from natural sea corals [133] and HA replaces aragonite whilst preserving its porous structure. The coral can be fully converted into hydroxyapatite and then coated with a sol-gel-derived apatite. This new material can be applied to bone graft applications where high strength requirements and longevity are pertinent [134,135].

When powder bioceramics are used for bone filling applications, they are usually mixed with a polymeric carrier matrix to avoid migration out of the implant region [136].

Both non-absorbable (polymethyl methacrylate [137], polyethylene [138] and polysulfone) and biodegradable (polylactic acid [139], polyglycolic acid, collagen, cellulose and starch [140-142]), polymeric matrices can be used, even if the non-biodegradability drastically reduces the HA crystal bioactivity.

Synthetic biodegradable biomimetic hydroxyapatite nano and micro microparticles exhibit excellent properties as bone filler biomaterial, such as biocompatibility, bioactivity, osteoconductivity, direct bonding to bone, etc., exciting new applications of HA in the fields of bone tissue engineering and orthopaedic therapies [143,144]. There are many synthetic strategies to produce HA microparticles, including wet producing, hydrothermal, electrochemical and ultrasonic mobilization methods, solgel and solid state synthesis. HA microparticles with different stoichiometry and morphology have been prepared and the effects of varying synthesis conditions on stoichiometry, crystallinity, morphology, surface properties, reactivity and bioactivity has been investigated [145-149] In order to optimize its specific biomedical applications, especially new bone formation and drug delivery function, the physical-chemical features which should be tailored in synthetic biomimetic HA microparticles are dimensions, porosity, morphology and surface properties [150-153].

The chemical and biological properties of HA microparticles are strictly linked to their dimensions, the regulation of which requires a high level of biological and chemical control at the nano scale. Thus, the recent trend in biomaterials research is focused on overcoming the limitations of calcium phosphates, precisely hydroxyapatite ceramics, and in improving their biological properties via exploring the unique advantages of nanotechnology [154]. The trend is shifting towards nanotechnology to improve the biological responses of HA, because nano-HA is a constituent of bone improving the biomaterial-bone interface. It has been established that biomimetism offers a unique approach to overcome many traditional materials shortcomings. Nanostructured biomimetic materials offer much higher performances than their larger particle sized counterparts, due to their large surface to volume ratio and unusual chemical/electronic synergistic effects. In addition, the surface adsorption properties of these materials led to applications in affinity chromatography [155], wastewater remediation [156] and drug delivery systems [149,157,158]. The surface functionalization of HA microparticles with bioactive molecules makes them able to transfer information to and to act selectively on the biological environment, and this represents a main challenge for innovative bone substitute materials. In this way HA microparticles will not only guarantee, for instance, either osteointegration or osteoinduction enhanced properties but they will also perform at the molecular level, by stimulating specific cellular responses.

Only in recent years scientists have begun to use biomolecules for the synergistic coupling of microparticles synthesis and functionalization. In fact, previous studies have limited the use of biomolecules as simple growth inhibitors of HA crystallization, rather than considering their use as a strategy to fine-tune the bioactivity of the microparticles [159,160]. Studies of the effect of biological molecules onto hydroxyapatite crystal growth have been related directly to physiological or pathological calcification processes. The exposure of biomaterials to plasma proteins, blood or biological fluids normally leads to the adsorption of blood proteins onto the biomaterial surface.

The adsorbed protein layer can further mediate additional biological responses, such as cell attachment and activation, and can create unpredicted perturbations to device operation [161-168].

Synthesis and characterisation of biomimetic carbonate-hydroxyapatite microparticles

Biomimetic carbonate-hydroxyapatite microparticles (CHA) have been synthesized with a nearly stoichiometric in bulk Ca/P molar ratio of about 1.6-1.7 and containing 4±1 wt% of carbonate ions replacing prevalently phosphate groups. CHA microparticles have been synthesized both about 100nm and 20 nm sized with an acicular and plate morphology respectively. TEM images of synthetic CHA microparticles 20 nm sized showing the plate shaped morphology and synthetic CHA microparticles 100 nm sized showing the acicular morphology are reported in Fig 6(A),(B) respectively.

CHA microparticles can aggregate in micro sized crystal clusters, whose dimensions increase prolonging maturation time in mother solution at constant temperature and stirring [169]. Powder X-ray diffraction patterns of plate shaped about 20 nm sized CHA microparticles and acicular shaped about 100 nm sized CHA microparticles (Fig. (7)(b,c)) respectively) show characteristic diffraction maxima of an apatite single phase (JCPDS 9-432). These X-ray diffraction patterns are compared with those collected for natural carbonate hydroxyapatite from deproteined dentine and enamel reported in Fig.(7)(a,d) respectively. The broadening of the diffraction maxima present in the X-ray diffraction patterns reported in Fig. (7)(a,b,c) indicate a relatively low degree of crystallinity, The degree of crystallinity of synthesized about 20 nm sized CHA microparticles with plate morphology and synthesized about 100 nm sized CHA microparticles with acicular morphology is 30% and 50% respectively.

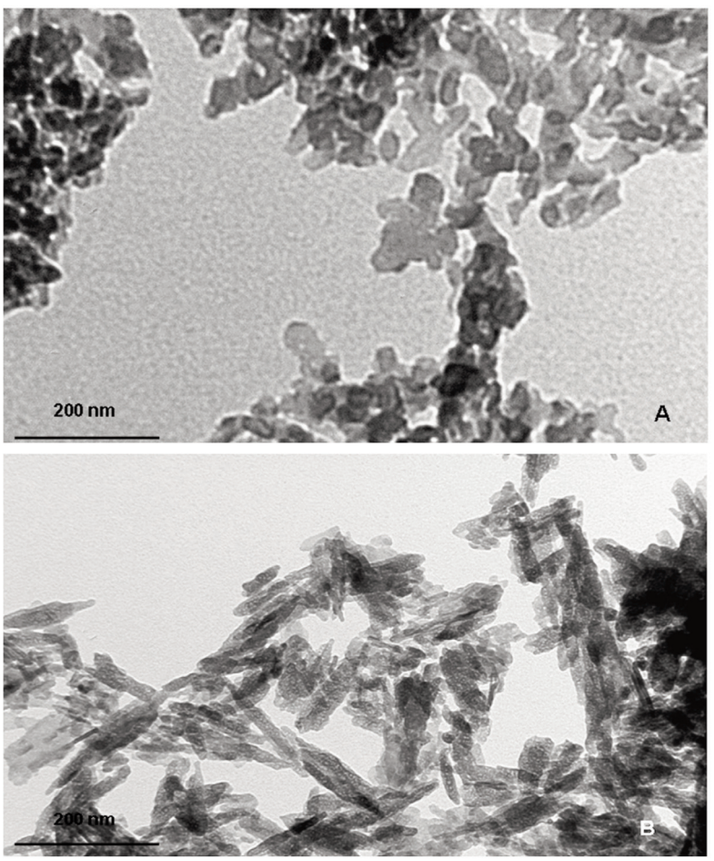


Fig.6 TEM images of synthetic CHA microparticles 20 nm sized showing the plate-shaped morphology (A), synthetic CHA microparticles 100 nm sized showing the plate-acicular morphology (B), (scale bar =200nm).

The crystallinity degree of about 20 nm sized CHA microparticles is very close to that one determined from the X-ray diffraction pattern of deproteined dentine natural carbonatehydroxyapatite (28%). Furthermore the crystallinity degree of natural hydroxyapatite of deproteined enamel is 70%. X-ray diffraction investigation reveal that the crystal structures of the synthesized CHA microparticles are very close to those observed for natural dentine.

The same similarity can be observed from the comparison of the FTIR spectra of synthesized CHA microparticles and natural apatite of deproteined dentine reported in Figure 6a and b respectively. In these spectra the characteristic absorption bands of phosphate and carbonate groups are clearly resolved. The absorption band at 1468 cm^{-1} is related to the carbonate group substitution to the phosphate one, while the shoulder at 1545 cm^{-1} can be considered the contribution of the carbonate group substituting the hydroxyl group in the apatite structure. This finding reveals that synthesized CHA microparticles not only contain a similar carbonate amount, but also, underline that the carbonate substitution to the phosphate and/or hydroxyl group

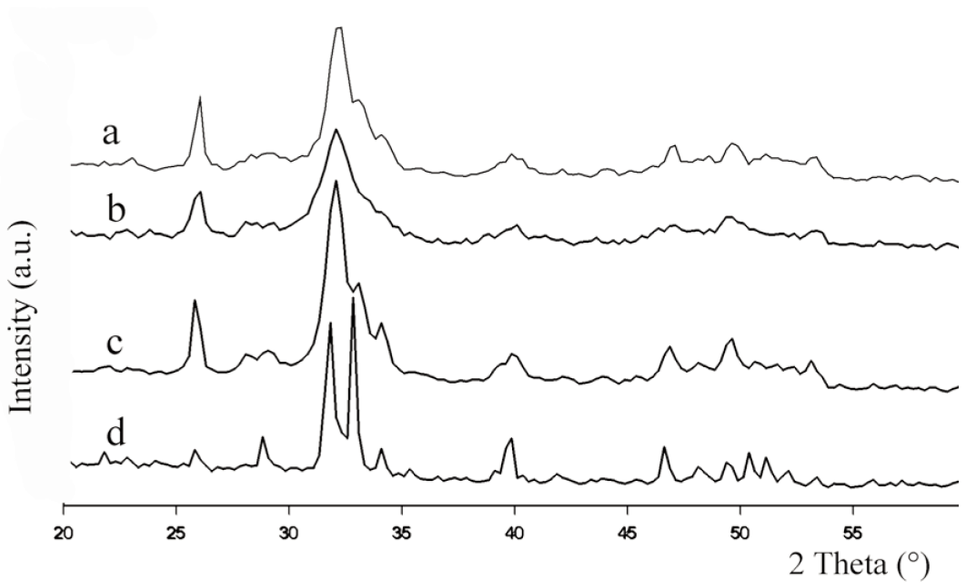


Fig.7 X-ray diffraction pattern of natural carbonate-hydroxyapatite from deproteinated dentine (a), synthetic CHA about 20 nm sized plate shaped microparticles (b), synthetic CHA about 100 nm sized plate-acicular shaped microparticles (c), and natural carbonate-hydroxyapatite from enamel (d).

is very similar in the synthetic and biological microparticles revealing that it can be considered a type B carbonate apatite.

A surface characterization of the synthetic carbonate-hydroxyapatite microparticles has been carried out in order highlight their surface chemical-physical characteristic which directly interfaces and reacts with exposed dental tissues. The ATR spectra of the synthetic about 20 nm and 100 nm sized CHA microparticles, reveal a 4% and 3% wt surface carbonate respectively. The consistent amount of surface % wt of carbonate present in synthetic CHA is appreciably higher than the surface % wt of carbonate present in enamel and dentine about 2% wt.

Specific surface area of 100 m^2g^{-1} and 80 m^2g^{-1} has been determined for 20 nm sized CHA microparticles with plate morphology and synthesized 100 nm sized CHA microparticles with acicular morphology respectively. These specific surface area values obtained for synthetic microparticles are only slightly lower than the 110 m^2g^{-1} obtained for biological microparticles.

The surface Ca/P molar ratio determined by XPS analysis for CHA microparticles and CHA microparticles micro-clusters do not reveal appreciable differences and result significantly lower than Ca/P molar ratio determined by ICP analysis in bulk indicating a surface calcium deficiency probably due to surface disorder. In fact the Ca/P molar ratios of 1.7 determined in bulk for synthetic CHA microparticles reduces to a value of 1.4-1.5 when determined on the microparticles surface by XPS analysis [170].

Teeth remineralization by biomimetic carbonate hydroxyapatite

The dentine remineralizing effect of sintetic biomimetic CHA nanometric crystal has been studied with a scanning electron microscopy putting a CHA nano microparticles slurry solution onto slices of dentine previously demineralized with ortophosphoric acid. The HA application shows the progressive closure of the tubular openings of the dentine with plugs within 10 minutes and a regeneration of a surface mineral layer within 6 hours (Fig. (8A-D)).

Biomimetic hydroxyapatite microparticles were demonstrated able to remineralize the surfaces of the dentine etched by orthophosphoric acid application and able to progressively occlude dentine tubules in few minutes till a regeneration of a layer of mineralized tissue within few hours. This remineralization rate seems to be compatible with the development of toothpastes with remineralizing effect and able to contrast dentine hypersensibility [171].

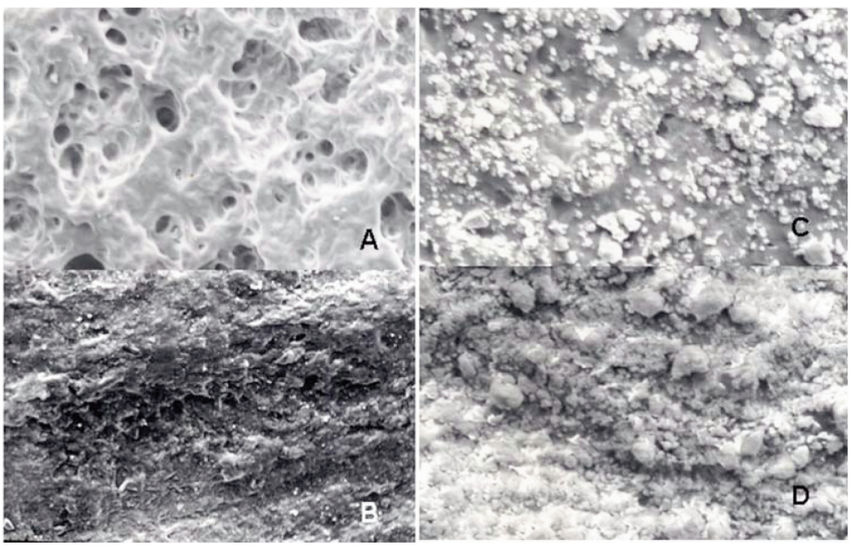


Fig.8 Scanning Electro Microscopy images showing the features of the dentine surface demineralised (A), to remineralized specimens after application of HA nanocristals slurry solution for 10 min (B), 1 h (C) and 6 h (D). The pictures show a progressive microparticles formation and consequent obliteration of dentinal patent tubules.

Scanning Electron Microscopic analysis allows investigating the morphology of both demineralized enamel and the features observed after remineralization procedures induced by biomimetic HA microparticles in vitro application. In fact after treatment for 10 minutes by aqueous slurry of synthetic 100 nm sized CHA microparticles, the surface of the demineralized enamel by ortophosphoric acid 37% for 1 minute, appears partially covered by the CHA phase and the interprismatic and prismatic enamel structure become not completely hidden. In contrast, the surface treated for 10 minutes by aqueous slurry of synthetic 20 nm sized CHA microparticles, the interprismatic and prismatic enamel structures appear to be covered by a thicker and more homogeneous apatitic layer [172]. This finding reveals an advantage of the 20 nm sized synthetic building block in respect to the 100 nm sized in producing an apatitic coating on the enamel surface.

XPS analysis of spectral features of the O 1s region of the enamel demineralized by ortophosphoric acid 37% for 1 minute compared with that of the enamel remineralized by synthetic 20 and 100 nm sized CHA microparticles for 10 minutes unequivocally confirm the presence of synthetic CHA at the surface of the treated enamel and the consequent validation of the enamel remineralization. The same finding is pointed out by the ATR spectrum of enamel treated for 10 minutes by synthetic 20 and 100 nm sized CHA microparticles, showing appreciable higher intensity of the characteristic absorption bands of carbonate ions (at 1420-1460 and 1680 cm^{-1}) in respect of the same absorption bands present in the demineralized enamel ATR spectrum, revealing that the surface of remineralized enamel is richer in carbonate than the natural one, like synthetic 20 and 100 nm sized CHA microparticles [172].

It has recently been revealed that the basic enamel building blocks are 20–40 nm. HAP microparticles and it has been suggested that the enamel repairing effect of HAP can be greatly improved if their dimensions can be reduced to the scale of the natural building blocks. Compared with conventional HAP and nano amorphous calcium phosphate (ACP), in vitro experimental results demonstrate the advantages of 20 nm HAP in enamel repairs. Scanning electron microscopy, confocal laser scanning microscopy, quantitative measurement of the adsorption, dissolution kinetics and nanoindentation show the strong affinity, excellent biocompatibility, mechanical improvement and the enhancement of erosion-free by using 20 nm particles as the repairing agent.

However, these excellent in vitro repairing effects cannot be observed when conventional HAP and ACP are applied. Clearly, 20 nm nano HAP share similar characteristics of the natural enamel building blocks, so that they may be used as an effective repairing material and anticaries agent.

Our current study highlights the analogies of nano building blocks of biominerals during biomedical applications, which provide a novel pathway for biomimetic repair [173].

Different enamel remineralization in vitro by toothpastes containing fluoride or biomimetic CHA microparticles micro-clusters

SEM analysis has been used to investigate the morphology of both demineralized enamel and the features observed after a remineralization process which utilises in vitro application of toothpastes containing fluoride or CHA micro-clusters constituted of microparticles 100 nm sized [170].

The surfaces of the teeth treated with fluoride (Fig. (9C)) were not consistently changed in respect to those of demineralized by ortophosphoric acid (Fig. (9B)). Actually both interprismatic and prismatic enamel structures still appear evident. On the contrary, after treatment of the enamel slabs with a toothpaste containing synthesized CHA micro-clusters constituted of microparticles 100nm sized the interprismatic and prismatic enamel structures appear to be completely hidden by a thick homogeneous apatitic layer (Fig. (9A)).

The XRD patterns collected on the surface of enamel slabs after treatment with CHA or fluoride tooth-pastes and water are reported in Figg. (10 b,c,d) respectively and compared with the XRD pattern (Fig. (10a) of CHA micro-clusters constituted of 100 nm sized microparticles utilized to prepare the used CHA tooth-paste. The XRD diffraction maxima recorded on the surface of enamel slabs treated with fluoride containing tooth-pastes appear slightly more sharpened than those obtained on the enamel etched slabs brushed only with water. This observation reveals an increased crystallinity degree probably due to a partial structural conversion of hydroxyapatite into fluoride substituted hydroxyapatite. On the contrary, the XRD pattern obtained on the surface of enamel slabs brushed with CHA containing tooth-paste shows the broadened diffraction maxima characteristic of the synthetic biomimetic CHA, revealing its presence on the enamel surface. The CHA not removed by brushing procedures suggests the formation of chemical bonds between the synthetic CHA micro-clusters constituted of 100 nm sized microparticles and natural enamel apatite microparticles. These bonds allow the formation of a persistent CHA coating on the enamel surface whose morphology was detected by SEM analysis.

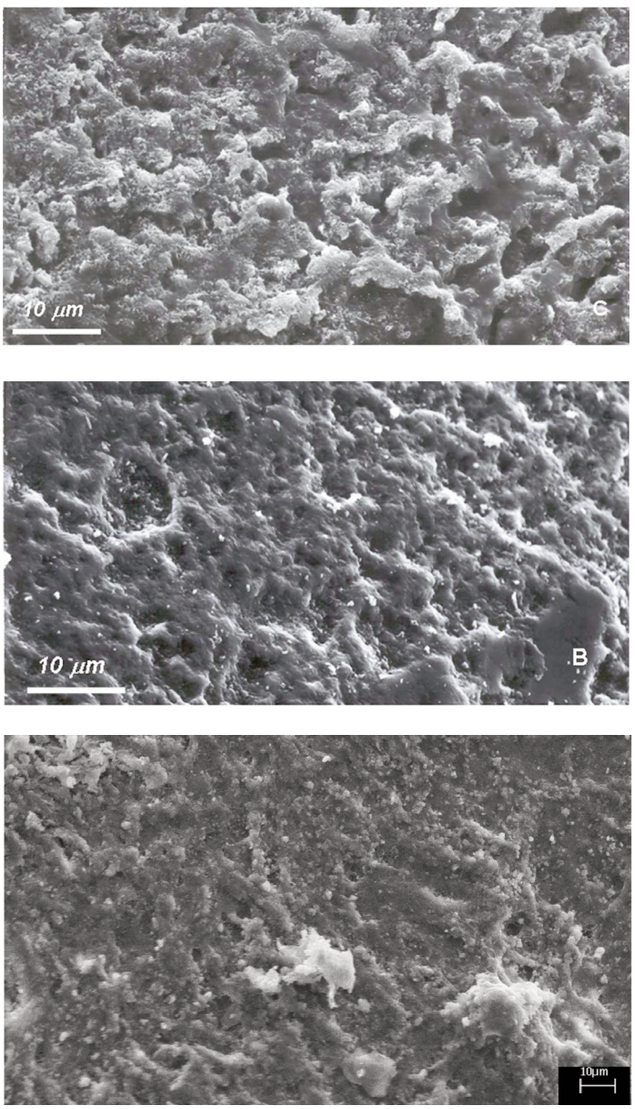


Fig. 9. SEM images of enamel after brushing treatment with: fluoride containing toothpaste (C), enamel surface after application ortophosphoric acid (B) and CHA containing toothpaste (A).

The surface Ca/P molar ratio determined by XPS analysis for demineralized enamel slabs before and after in vitro remineralization by brushing with toothpastes containing fluoride or CHA have been compared with the Ca/P molar ratio of CHA micro-clusters constituted of 100 nm sized microparticles. The enamel surface Ca/P molar ratio practically does not

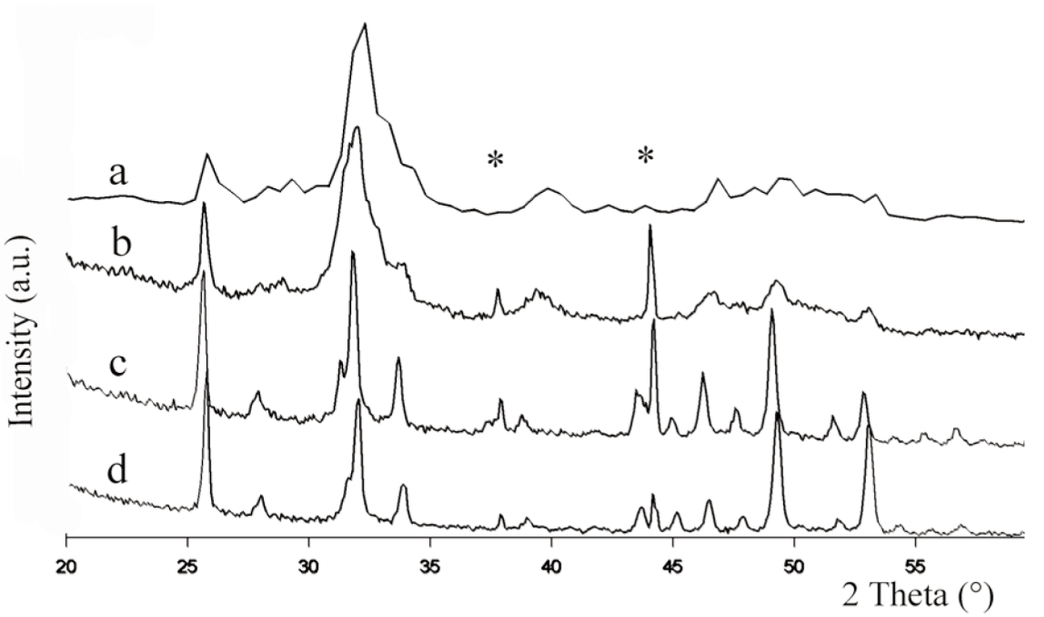


Fig. 10. XRD patterns of enamel after brushing treatment with: water (d), fluoride containing toothpaste (c), CHA microclusters containing toothpaste (b) and XRD patterns of synthetic CHA microclusters (a). * indicates Al holder diffraction maxima.

change before and after the brushing treatment with tooth pastes containing fluoride. This finding reveals how the only structural modification of enamel hydroxyapatite induced by fluoride is restricted to a partial hydroxyl group replacement by fluoride ions without affecting appreciably the Ca and phosphate structural network. On the contrary, enamel slabs after the brushing treatment with the toothpaste containing synthesised CHA micro-clusters of 100 nm sized microparticles the enamel slabs exhibit a surface Ca/P molar ratio very close to that of the synthetic CHA micro-clusters of 100 nm sized microparticles. The results highlight that biomimetic nano sized CHA microparticles produce an apatite coating deposition on the enamel surface. An advantage of the 100 nm sized synthetic building block respect the 20 nm sized in binding on to the enamel surface has been appreciated, but it can be ascribed to a different and more suitable micro-cluster aggregation.

This coating is much less crystalline than native enamel apatite, and consists of a new apatitic mineral deposition which progressively fills the scratches and pits. On the contrary, the surface remineralisation observed on the specimens treated with fluoride contained in toothpaste, is mainly based on chemical-physical enamel apatite surface modifications rather than on the formation of a new mineral deposition.

The documented CHA biomimetic coating formation, is the first remineralization process corresponding to a real new mineral apatitic deposition in the demineralized area of enamel surface.

Current and future developments

From some decades the teeth health care has been committed to the fluoride effect on hydroxyapatite. Fluoride interacts with hydroxyapatite promoting the conversion into fluorapatite, hich is less soluble and more mechanically resistant, but also more brittle than hydroxyapatite.

The action of fluoride on the surface enamel apatite appears ideal to prevent demineralization due to acid food and drinks and to contrast plaque damage. A lot of scientific works support the fluoride positive action for teeth health care and consequently a lot of fluoridated oral care products have been patented till nowadays and in some countries drinking water has been enriched with fluoride.

Fluoride is not essential for human growth and its content in the body is not under physiological control. Adsorbed fluoride is rapidly distributed in the body by circulation and is retained only in calcified tissues. In the last decades dangerous effects in human health have been ascribed to the daily fluoride amount ingest-

ed and many studies have put in evidence its high risk, especially for children, to get fluorosis and bone diseases in old people. European Food Safety Authority (EFSA) Scientific Panel has advised about the risk of fluoride intake from ingested oral care products and toothpastes can be fluoridated with a maximum level of 1500 mg/Kg. In spite of this knowledge, fluoride continues to be widely used in orthodontia and dental applications to prevent enamel surface demineralization and represents the main component in toothpastes claiming remineralization effects. If nowadays fluoride represents the unique agent to contrast caries, plaques and demineralization we may probably ascribe this situation to the expensive cost of odontoiatric products containing hydroxyapatite. In spite of this, hydroxyapatite is commonly considered the main synthetic biomaterial as bone filler and substitute and only few daily use products for teeth health care have been patented. Too expensive is the hydroxyapatite cost to be consistently utilised as an active agent in toothpastes or mouth washes. In fact, almost all the patents previously reported and highlighting the ability of hydroxyapatite to remineralize enamel and dentine, propose different compositions containing calcium and phosphate ions which can produce hydroxyapatite on teeth if mouth pH induces this crystallization and frequently are obliged to insert a low amount of fluoride in order to promote the apatite crystallisation. Small amounts of industrial hydroxyapatite are associated to other cheaper components and submitted to milling to improve hydroxyapatite surface area and reactivity. Only recently the development of nanotechnologies has opened new opportunities in obtaining cheap hydroxyapatite micro-nano particles by the "bottom up" methods.

These hydroxyapatites are surface nanostructured and have higher surface area and consequently higher reactivity allowing them to bing to enamel and dentine apatite producing on enamel a biomimetic coating, contrasting plaque formation and sealing dentine tubules annulling hypersensibility.

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