Biofunctional Properties of Caseinophosphopeptides in the Oral Cavity

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Key Words

Caries · Caseinophosphopeptides-amorphous calcium phosphate · Demineralisation · Erosion · Remineralisation

Abstract

Caseinophosphopeptides (CPPs), bioactive peptides released from caseins, have the ability to enhance bivalent mineral solubility. This is relevant to numerous biological functions in the oral cavity (promotion of tooth enamel remineralisation, prevention of demineralisation and buffering of plaque pH). Therefore, CPPs may play a positive role as prophylactic agents for caries, enamel erosion and regression of white spot lesions. Most in vitro and in situ studies demonstrate strong evidence for the bioactivity of CPPs in the oral cavity. Nevertheless, relatively little is known concerning their use as adjuvants for oral health and more particularly regarding their long-term effects on oral health.

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1. Introduction

1.1. The Prevalence of Dental Caries

The prevalence of dental caries represents a public health issue in many countries. Indeed, dental caries and periodontal diseases have been described as the most

prevalent or widespread condition within humans [Petersen and Kwan, 2009]. It has been reported that 90% of the world's population will experience caries at least once in their life [Marchisio et al., 2010]. In the USA for example, it is estimated that 90% of late adolescents and young adults are affected by caries while 94% of adults have coronal caries [Chen and Wang, 2010]. It has been proposed that early detection both in children and adults could allow a better management of caries [Pitts and Wefel, 2009]. Dental caries is defined as the destruction of tooth tissue by the action of oral micro-organisms. The first step of the process involves demineralisation of tooth hard tissue by organic acids produced from fermentable carbohydrates by cariogenic bacteria located in dental plaque [Cross et al., 2007a; Reynolds, 2008b, 2009; Elsayad et al., 2009; Chen and Wang, 2010; Featherstone et al., 2011]. Bacteria can colonize the tooth surface and adhere to the pellicle through adhesion-receptor interactions, resulting in the formation of biofilms [Hannig and Hannig, 2010]. A biofilm, which is defined as a spatial organisation of a microbial community in a polymer matrix (originating from the bacteria and saliva), adheres onto the tooth surface [Marsch et al., 2011]. These biofilms are typically referred as dental plaque. Dental plaque consists of different species of bacteria and a combination of polysaccharides, proteins and DNA [Chen and Wang, 2010]. Demineralisation of tooth enamel is caused by dissolution of apatite crystals [Cross et al., 2006]. This in turn

can lead to the appearance of lesions at the tooth surface. Caries incidence has significantly regressed in recent years following the utilisation of non-invasive interventions including the use of therapeutic agents such as fluorides to combat enamel demineralisation caused by bacteria located in dental plaque [Cross et al., 2006; Chen and Wang, 2010; Peters, 2010; Featherstone et al., 2011; Pradeep and Prasans, 2011]. Nevertheless caries is still a concern in some population groups including infants and adolescents or people who do not use oral hygiene products [Rose, 2000; Cross et al., 2007a; Rahiotis et al., 2008; Petersen and Kwan, 2009]. The prevalence of dental caries has been associated with poor living conditions, lifestyle (inadequate diet, tobacco, alcohol, oral hygiene) and the availability of oral health services [Petersen and Kwan, 2009]. The World Health Organisation has defined some priorities to decrease the incidence of dental caries worldwide, these include the increased utilisation of fluorides, a targeted improvement of oral hygiene in different age groups, development of oral health policies and promotion of oral health research [Petersen and Kwan, 2009]. Nevertheless, the utilisation of excessive amounts of fluoride can cause pathologies such as fluorosis during tooth development of young children. Fluorosis is caused by the ingestion of excessive amounts of fluoride, which results in a hypomineralisation of the tooth enamel and can cause the appearance of white lines or brown stains depending on the severity of the symptoms [Reynolds, 1998; Wong et al., 2010]. Children of 9 years who received a fluoride daily intake of 0.05 mg F/kg until the age of 48 months had no caries and no fluorosis. Below this value, children developed caries and above it, they could develop fluorosis [Warren et al., 2009]. Combinations of fluoride with other remineralising agents could help combat the development of dental caries in children and avoid the development of pathologies such as fluorosis.

1.2. The Prevalence of Dental Erosion

Besides caries, dental erosion is another important issue in oral health. In recent times while there appears to be a decline in dental caries prevalence [Borges et al., 2011], more attention is now being given to dental erosion [Tantbirojn et al., 2008] as an increase in its prevalence has been observed [Ranjitkar et al., 2009a; Wang et al., 2011]. Erosion is the consequence of the contact of teeth with low pH solutions (pH 1–3), which occurs during the consumption of acidic foods and fluids, or when gastric

fluids get into the oral cavity [ten Cate, 1999; Lussi, 2009; Panisch and Poolthong, 2009; Hannig and Hannig, 2010; Wegehaupt and Attin, 2010; Barbour et al., 2011]. In a recent review, Barbour et al. [2011] described low pH as the main factor in erosion and values below pH 5.0 have been reported as the threshold pH for erosion detection. Erosion can also be associated with parameters such as abrasive conditions during teeth cleaning or physiological deficiencies such as low saliva flow [Lussi, 2009]. Enhancement of the extent of tooth remineralisation has been proposed as a means to prevent dental erosion [Wegehaupt and Attin, 2010].

1.3. Potential of Caseinophosphopeptides as Natural Remineralising Agents

Caseinophosphopeptides (CPPs) are casein-derived phosphorylated peptides which have binding and solubilizing properties for a wide range of minerals such as calcium, magnesium and iron. In addition, CPPs may bind and solubilise other trace elements, such as zinc, barium, selenium, nickel, cobalt and chromium [FitzGerald, 1998]. CPPs have also been associated with improved dietary bioavailability of bivalent cations and as a consequence they may play a major role in modulating mineral uptake and bone formation [Meisel and Frister, 1988; FitzGerald, 1998]. Therefore, the utilization of CPPs to improve bone health has been the subject of much research [FitzGerald, 1998; Gueguen and Pointillart, 2000; Tulipano et al., 2010]. CPPs have also been associated with an improvement in oral health, notably for their role in promoting remineralisation of dental enamel, thereby combating the development of dental caries [Reynolds, 1997b; FitzGerald, 1998; Elsayad et al., 2009; Rehder Neto et al., 2009]. There has been a large interest in the potential applications of CPPs over the last 30 years, following increased public health awareness related to the consequences of poor bone health and dental pathologies such as caries. With recent developments in minimally invasive treatments, the interest in natural remineralising agents such as CPPs are becoming more relevant to dentistry [Borges et al., 2011; Gupta and Prakash, 2011]. As a consequence, CPPs have been commercially developed as natural ingredients arising from milk proteins mainly for their mineral binding properties [Reynolds, 1998]. A significant level of potential intellectual property has been devoted to the discovery of new CPP sequences or the isolation and development of new CPP applications in the area of oral hygiene and bone health [Reynolds et al.,

α_{s1} -casein f(59-79)5P

Gln-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys

 β -casein f(1-25)4P

Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg

Fig. 1. Peptide sequence of tryptic CPPs from α_{s1} -casein f(59-79)5P and β -casein f(1-25)4P highlighting the 'acidic motif' (fragment underlined).

2009; Reynolds, 2010]. Nevertheless, some conflicting evidence exists in relation to the efficacy of these biofunctional peptides.

The aim of this review is to critically assess current literature in relation to the role of CPPs in the remineralisation of teeth. Evidence for bioactivity of CPPs in the oral cavity will be discussed presenting information obtained in different studies carried out in vitro, in situ and in vivo. Special attention will be given to human intervention trials which have been performed to address the contradictions in relation to the bioactive potential of CPPs as remineralising agents in the oral cavity. The market situation will also be described outlining currently available CPP-containing products along with an overview of the patent terrain.

2. Caseinophosphopeptides

2.1. Structure of CPPs

CPPs correspond to peptide fragments which are rich in clusters of phosphorylated seryl (and occasionally threonine) residues. CPPs represent approximately 10% (w/w) of the primary sequence of the caseins [Swaigood, 1982; Cross et al., 2006, 2007a]. Caseins are phosphorylated during milk biosynthesis via the activity of specific kinases present in the mammary gland [FitzGerald, 1998]. The number of serine/threonine phosphate groups may be influenced by genetic polymorphism of the proteins. Therefore, different levels of phosphorylation may be observed in the individual caseins as follows: (1) α_{s1} -casein: 8–9 phosphate groups; (2) α_{s2} -casein: 10–13 phosphate groups; (3) β -casein: 5 phosphate groups; (4) κ -casein: 1–2 phosphate groups.

The two most studied CPPs originate from α_{s1} - and β -casein. Both of these CPPs [α_{s1} -casein f(59-79)5P and

β -casein f(1-25)4P] contain a specific sequence known as the 'acidic motif'. The 'acidic motif' consists of 3 serine phosphate groups followed by two glutamic acid residues (fig. 1). At neutral pH, the 'acidic motif' is a highly charged region and this has been linked with the ability of CPPs to bind minerals (Ca^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , Se^{2+} , etc.) [Rose, 2000]. Seryl-phosphate groups are the main binding sites for calcium [Kitts, 2006]. Nevertheless, characterisation of the calcium binding constants to CPPs suggest that carboxylate groups may also be involved in calcium binding [Mekmene and Gaucheron, 2011]. Other CPP fragments such as α_{s2} -casein f(1-21)4P and α_{s2} -casein f(46-70)4P have also been described in the binding of calcium [Reynolds, 1998; Cross et al., 2006; Kitts, 2006; Cochrane et al., 2010]. These phosphorylated peptide sequences can be released following hydrolysis of casein substrates with enzymes such as trypsin [Reynolds et al., 1995; Rose, 2000]. The high charge content of CPPs which allows them to bind bivalent cations forming soluble complexes also appears to make them resistant to further hydrolysis [Vegarud et al., 2000; Silva and Malcata, 2005].

2.2. Formation of CPPs

CPPs are encrypted within the primary sequence of caseins and must be released in order to become active [FitzGerald, 1998].

2.2.1. Release of CPPs from Milk Protein Substrates

A number of different release approaches exist as follows:

- In vitro hydrolysis following incubation with proteolytic activities from mammalian, microbial and plant sources or via the utilisation of physical and/or chemical processes such as ultrasonic, microwave and chemical treatments.

Table 1. Example of studies reporting the formation of CPPs in vivo following the ingestion of milk and dairy products [adapted from FitzGerald, 1998]

Diet	System (in vivo)	CN origin	Reference
Casein	rat (small intestine)	n.d.	Naito et al. [1972]
Casein	minipig (jejunal fluid)	α_{s1} -CN	Meisel and Frister [1988]
Milk/yoghurt	human (stomach and duodenum)	α_{s1}/β -CN	Chabance et al. [1998]
Milk/CPPs	human (ileostomy fluid)	n.d.	Meisel et al. [2003]

CN = Casein; n.d. = not determined.

- Hydrolysis by bacterial proteolytic/peptidolytic activities during the manufacture of fermented dairy products. Such hydrolysis may also occur in vivo following the action of intestinal microflora found in the human body.
- In vivo digestion via the combined action of gastric and pancreatic hydrolases during gastrointestinal transit following oral ingestion. Subsequently, epithelial and serum peptidases may mediate further degradation assuming transport across the intestinal mucosa and transfer into the serum. Several studies performed in mammals including humans, pigs and rats have demonstrated the in vivo formation of CPPs following ingestion of milk and dairy products (table 1). CPPs have been found in the distal small intestine of the rat, and in the stomach, duodenum and distal ileum of human subjects [Chabance et al., 1998; Meisel et al., 2003; Hartmann and Meisel, 2007] following the consumption of dairy ingredients and products.

Most of the commercially available CPP preparations are released from casein using the first approach (i.e., enzymatic hydrolysis using proteolytic activities). Different pancreatic endoproteases have been used to cleave casein substrates to release clusters of phosphorylated seryl residues [McDonagh and FitzGerald, 1998]. Amongst these enzymes, trypsin is the most commonly used [FitzGerald, 1998; Vegarud et al., 2000]. Other enzymes from microbial and plant sources have also been used to release CPPs from sodium caseinate or other casein substrates [Adamson and Reynolds, 1996; FitzGerald, 1998; McDonagh and FitzGerald, 1998; Kitts, 2006]. A general protocol for CPP production and isolation from sodium caseinate is presented in figure 2 [Reynolds, 1991]. After tryptic hydrolysis of the sodium caseinate substrate, the pH is adjusted to isoelectric pH of casein to precipitate unhydrolysed caseins [Reynolds et al., 1994]. CPPs remain in solution and can be separated from the precipi-

Fig. 2. Schematic representation of the extraction procedure of CPPs [adapted from Reynolds, 1991].

Locus	Target	Bioactivity
Mouth	Teeth	Antibacterial action and remineralisation
Gastro-intestinal tract	Bioavailability	Mineral solubility
Body	Bones	Remineralisation

Fig. 3. The possible sites of action of CPPs in vivo.

tated caseins using a centrifugation step. A calcium salt (BaCl_2 or CaCl_2) is added to the supernatant to form Ca^{2+} complexes with the phosphorylated peptides. At pH 3.5 combination of calcium and ethanol allows selective precipitation of the CPP sequences with the acidic motif [Reynolds et al., 1994]. Following this step, different pH adjustments are carried out to precipitate or resolubilise CPPs in order to separate them from other casein-derived peptides. Sulphuric acid is then used to precipitate BaSO_4 or CaSO_4 . After a centrifugation step, a supernatant containing CPPs is obtained and can be dialysed for further purification. Spray-drying of this product allows obtaining a stable powder.

2.2.2. Stability of CPPs during Enzymatic Hydrolysis

CPPs are formed and may be degraded during enzymatic hydrolysis. When performing casein hydrolysis with porcine PancreatinTM, the formation of CPPs containing the 'acidic motif' [Ser(P)-Ser(P)-Ser(P)-Glu-Glu] in the early stages of hydrolysis has been demonstrated (i.e., 10 min hydrolysis). However, these CPPs were totally degraded after 6-hour hydrolysis. Su et al. [2007] proposed a kinetic approach for casein hydrolysis in order to optimise the yield of CPPs. After 1-hour hydrolysis of casein with porcine Pancreatin, 27% of the CPPs identified contained the binding site for bivalent cations, i.e. Ser(P)-Ser(P)-Ser(P)-Glu-Glu [Su et al., 2007]. The optimum degree of hydrolysis may differ depending on the CPP sequence of interest. For example, α_{s2} -casein f(1-44)4P and α_{s2} -casein f(1-44)5P had a maximum yield at a degree of hydrolysis of 3%, for α_{s2} -casein f(44-81)4P the optimum degree of hydrolysis was 6% while for α_{s2} -casein f(46-80)4P the maximum yield was reached at a degree of hydrolysis of 12% [Su et al., 2007].

The stability of CPPs following heat treatment at different pH values has been studied [Zhu and FitzGerald, 2010]. A heat treatment is generally applied following en-

zymatic hydrolysis of casein to heat-inactivate the enzyme. The effect of the heat treatment (75°C, 45 min) at different pH values (6.0, 7.0 and 8.0) on the stability of CPPs has been studied. Mass spectrometric analysis of the trypsin hydrolysates (3-hour hydrolysis of sodium caseinate with tosyl phenylalanyl chloromethyl ketone-treated trypsin) subjected to the different heat treatments revealed some modifications in the CPP sequences. Differences between the heat-treated and the non-heat-treated hydrolysates mainly came from dephosphorylation and oxidation of the CPP sequences identified. Heat treatment at pH 7.0 gave the highest number of CPPs with phosphorylated sequences. Additionally, the sequences identified following heat treatment at pH 7.0 were more similar to those identified in the non-heat-treated hydrolysate [Zhu and FitzGerald, 2010]. These results suggest that the heat-inactivation parameters employed after hydrolysis of casein play an important role in the stability of CPPs and will therefore affect bioactivity of the product obtained.

2.2.3. Formation of CPP-Amorphous Calcium Phosphate

CPPs may act at different sites in vivo (fig. 3). Following ingestion, their first target is in the mouth where they may play a role in the repair of teeth lesions. Amorphous calcium phosphate [ACP, $\text{Ca}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$] is the precursor of hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$]. In the presence of water, ACP can be converted into hydroxyapatite [Boskey and Posner, 1973]. At the tooth surface, calcium and phosphate precipitate in the stable format of hydroxyapatite [Skrtic et al., 2003]. Combinations of CPP and ACP have been utilised for remineralisation of the enamel surface and for reducing the rate of enamel decalcification. CPPs in tryptic digests having the acidic motif have been reported to stabilize amorphous calcium phosphate and to exhibit anticariogenic properties [Adamson and Reynolds, 1996; Meisel et al., 2003; Kitts, 2006]. CPP-ACP formation has been described [Walsh, 2009] where CPPs are aggregated with calcium phosphate and then purified by ultrafiltration, the nanocomplexes are formed at a pH range between 5.0 and 9.0. The amount of calcium phosphate bound to the CPP increases with pH until an equivalent weight of calcium phosphate is bound to the CPP [Cross et al., 2007a; Walsh, 2009]. The protocol for the manufacture of CPP-ACP and CPP-amorphous calcium fluorophosphate (ACFP) complexes has been described in the patents filed by Reynolds [2008c, 2011] (fig. 4). In this process CaCl_2 was slowly added to the CPPs. An NaOH solution was then added to the previ-

Fig. 4. Schematic representation of CPP-ACP complex manufacture [adapted from Reynolds, 2008c].

ous mixture following each addition of CaCl_2 to set the pH between values ranging from 7.0 to 5.0. Then an Na_2HPO_4 solution was slowly added to form CPP-ACP complexes. For the manufacture of CPP-ACFP complexes, an extra step consisted of the incorporation of NaF. The CPP-ACP and CPP-ACFP complexes formed were soluble in water and therefore, they could be separated from the other components (other casein-derived peptides and salts) on a molecular weight basis. Slight variations to the previous protocol have been described for the preparation of CPP-ACFP complexes, where the pH was regulated to 9.0 and the microfiltration step was replaced by dialysis through a 1-kDa membrane [Cross et al., 2004]. CPP-ACP complexes have been reported to be composed of a maximum of 6 peptide chains with a unit formula depending on the peptide involved: $[\alpha_{s1}\text{-CN}(59\text{--}79) (\text{ACP})_7]_6$ and $[\beta\text{-CN}(1\text{--}25) (\text{ACP})_8]_6$ [Cross et al., 2007a]. The average hydrodynamic radii of the $[\beta\text{-CN}(1\text{--}25) (\text{ACP})_8]_6$ complex has been estimated using structural analyses (^1H NMR) as 1.526 ± 0.44 nm at pH 6.0 and 1.923 ± 0.082 nm at pH 9.0, respectively [Cross

et al., 2007a]. Clusters of α_{s1} -casein f(59–79)-ACFP are reported to have a radius of about 2.12 nm [Cross et al., 2004]. These clusters are smaller than the CaF_2 clusters which were developed as anticaries agents using two liquid nozzle spray-drying technology. These clusters had an estimated average diameter of 41 nm [Sun and Chow, 2008].

Teeth are composed of four tissues which from the outer to the inner compartment consist of enamel, dentin, cementum and the pulp, respectively. The enamel and dentin are the sites of primary dental caries [Borges et al., 2011]. Therefore, these two tissues are sites for teeth remineralisation by CPP-ACP. In the oral cavity, CPP-ACP can act on the teeth at different levels as follows:

- The enamel and dentin: where binding of ACP to the hydroxyapatite occurs.
- The plaque: CPP-ACP can diffuse into dental plaque and display a buffering capacity which counteracts the pH drop caused by acidogenic bacteria [Adamson and Reynolds, 1996; Chen and Wang, 2010]. An additional role of CPP-ACP relates to an increase in calcium content in the plaque which results in a greater remineralisation of teeth lesions. CPPs have also been reported to localise ACP in dental plaque and prevent enamel from demineralisation [Adamson and Reynolds, 1996].

2.3. Commercial Applications of CPPs

Numerous patents concerning the manufacture of CPPs have been developed. Most applications relate to bovine milk as a source of CPPs (table 2). A wide variety of starting substrates have been used, these vary from whole milk to other substrates with higher protein contents such as sodium caseinate. The patent literature describes similar strategies as those outlined in the scientific literature to release CPPs from milk protein substrates [FitzGerald, 1998]. Enzymatic hydrolysis appears to be the most frequently used technique to release CPPs, with a predominance of pancreatic activities such as trypsin being described in various patents. Mention of activities originating from plants such as papain (plant proteinase preparation extracted from papaya) is also made in two of the patents (table 2).

As CPPs represent approximately 10% (w/w) of the caseinate substrate [Reynolds, 1998], most of the patenting activity has been carried out in the development of extraction techniques for CPPs from dairy protein hydrolysates. Such patents account for almost half of all the specifications described in table 2. A large number of these

Table 2. Summary of patents describing the production, isolation and use of CPPs in nutrition and oral hygiene applications

Patent	Title	Component	Methods of production and extraction	Benefit area	Target	Patent category
US4,361,587 [Brule et al., 1982]	Phosphopeptides from casein-based material	Phosphopeptides originating from skimmed milk	Enzymatic hydrolysis (Pancreatin in the form of a natural pancreatic extract or mixtures of trypsin and α -chymotrypsin) of skimmed milk Membrane separation (UF) and disaggregation to isolate phosphopeptides from the proteolytic enzyme and the non-phosphorylated peptides	Salts of phosphopeptides, which have dietetic uses	Humans (enteral formulas)	E
US4,495,176 [Brule et al., 1985]	Phosphopeptides from casein-based raw material	CPP (organophosphorated salts) from NaCN or paracasein	Enzymatic hydrolysis (Pancreatin in the form of a natural pancreatic extract, or a mixture of trypsin and α -chymotrypsin) of NaCN or paracasein Membrane separation (UF) and aggregation (bivalent cation salt) to isolate phosphopeptides from the proteolytic enzyme and the non-phosphorylated peptides	Phosphopeptide salts (calcium and/or magnesium and/or oligoelements such as iron and zinc) dietetic uses	Humans (enteral formulas)	E
US4,816,398 [Brule et al., 1989]	Casein phosphopeptide composition	Casein phosphopeptide composition (<4% phenylalanine, tyrosine and tryptophan, >8% and <20% serine, ratio of Ca+Mg+P/total nitrogen >0.2 and free amino acids <3%)	Enzymatic hydrolysis of casein (Pancreatin in the form of a natural pancreatic extract or a mixture of trypsin and α -chymotrypsin) Membrane separation (UF) and aggregation with a bivalent cation salt to isolate phosphopeptides	Aliment for dietetic or therapeutic nutrition or medicament	Humans	E
US5,028,589 [Brule et al., 1991]	Casein phosphopeptide salts	Casein phosphopeptide salts of calcium, magnesium or both (<4% by weight of aromatic amino acids, >8% and <20% by weight serine; <3% free amino acid; total calcium, magnesium and phosphorus to total nitrogen >0.2)	NaCN or paracasein enzymatic hydrolysis (Pancreatin in the form of a natural pancreatic extract or a mixture of trypsin and α -chymotrypsin) Membrane separation (UF) and aggregation with a bivalent cation salt to isolate phosphopeptides	Aliment for dietetic or therapeutic nutrition and medicines	Humans (enteral formulas)	E
US5,216,123 [Brule et al., 1993b]	Non-phosphorylated peptides from casein-based material	Non-phosphorylated peptides and phosphopeptides	Enzymatic hydrolysis (Pancreatin in the form of a natural pancreatic extract or a mixture of trypsin and α -chymotrypsin) Membrane separation (UF) and aggregation with a bivalent cation salt to isolate the non-phosphorylated fraction	Phosphopeptides form salts, which have dietetic uses, with macroelements such as calcium and/or magnesium and/or oligoelements such as iron and zinc	Humans (enteral formulas)	E
US5,219,735 [Brule et al., 1993a]	Non-phosphorylated peptides from casein-based material	Non-phosphorylated peptides and phosphopeptides	Casein-based material (skimmed milk) enzymatic hydrolysis with Pancreatin Membrane separation (UF) and disaggregation (acidification) to isolate the non-phosphorylated fraction	Phosphopeptides form salts, which have dietetic uses, with macroelements such as calcium and/or magnesium and/or oligoelements such as iron and zinc	Humans (enteral formulas)	E

US5,334,408 [Brule et al., 1994b]	Nutrient composition containing non-phosphorylated peptides from casein-based material	Non-phosphorylated peptides	Casein-based material (NaCN, caseinomacropetide) enzymatic hydrolysis (Pancreatin in the form of a natural pancreatic extract or a mixture of trypsin and chymotrypsin) Membrane separation (UF) and aggregation with a bivalent cation salt to isolate the non-phosphorylated fraction	Non-phosphorylated peptides (compositions for providing nutrition) and phosphopeptides salts (dietetic uses)	Nutrition formulas (enteral formulas)	E
US5,352,476 [Brule et al., 1994a]	Nutrient composition containing non-phosphorylated peptides from casein-based material	Non-phosphorylated peptides	Casein-based material (skim milk) enzymatic hydrolysis (Pancreatin, chymotrypsin, trypsin) of a casein-based material (skim milk) Membrane separation (UF) and disaggregation (acid or calcium complexant) to isolate the non-phosphorylated fraction	Non-phosphorylated peptides and phosphopeptides useful as alimentary products or medicaments	Nutrition compositions	E
US5,290,685 [Koide et al., 1994]	Method for separation and concentration of phosphopeptides	Method for separating and concentrating acidic peptides, especially phosphopeptides having a phosphoserine residue	Casein (acid casein, sodium caseinate and calcium caseinate) enzymatic hydrolysis with protease (trypsin, pepsin, chymotrypsin or papain) Cross-linkage of the CPP on chitosan beads Desorption of the CPP at pH <1.5 or >5	CPPs for use as ingredients in food materials, functional foods, nutrients, etc.	-	E
US5,780,593 [Lihme et al., 1998]	Method of isolating biomolecules by ion exchange	Ion-exchange method for the isolation of CPPs	Casein hydrolysates (trypsin) Membrane separation (UF) followed by anion exchange chromatography	Prevention of osteoporosis (food, feed, health-care product, cosmetic or pharmaceutical products)	Adults	E
US5,405,756 [Naito et al., 1995]	Transparent acid drink containing acid-soluble casein phosphopeptide	Transparent, storage, stable acidic (pH ≤3) beverage or other alimentary product containing a mixture of α- and β-CPPs	Casein (NaCN) trypsin hydrolysis Precipitation of CPPs (acid, Fe ²⁺ , Ca ²⁺ , BaCl) or extraction with active carbon Addition of CPP to a mixture to produce a beverage or product having beneficial solubilising effects on calcium	Food to increase absorption by the human body of calcium and iron for growing infants and children, treatment of anaemia (women), and osteoporosis (aged people), lactose intolerants	Infants, women and old people	F
US7,060,472 [Holt, 2006]	Calcium phosphate nanoclusters and their applications	Particles of calcium phosphate nanoclusters coupled with CPP, with high calcium concentrations	Whole casein enzymatic hydrolysis (protease XIV and papain type IV) or α _{s1} -casein enzymatic hydrolysis (tryptic digest) Membrane separation (UF)	Treatment of tooth demineralisation	Broad	F
US7,968,513 B2 [Iscovich et al., 2011]	Pharmaceutical compositions comprising casein derived peptides and methods of use thereof	Pharmaceutical compositions with casein-derived peptides in the format of a clear sterile solution	Generation of casein hydrolysates with trypsin or pancreatic extracts. Microfiltration of casein hydrolysate yielding a mixture of low-molecular weight (1–5 kDa) peptides derived from β-, α _{s1} - and α _{s2} -casein	Therapeutic amounts of CPPs for the management of lactating animals to decrease length of the dry period, increase milk yield and hygiene, and prevent mammary gland infection	Lactating animals (dairy herds) and humans	F
US6,180,761 [Han and Shin, 2001b]	Casein from Korean cattle	New CPP obtained from a novel casein	Casein having a novel amino acid sequence (isolated from the milk of Korean cattle with a β-casein A1 H genotype) Enzymatic hydrolysis with trypsin, no further extraction of the CPP formed	Solubilisation and promotion of mineral absorption (infant formulas, lactose intolerance, osteoporosis and anaemia)	Infants and adults	S

Table 2 (continued)

Patent	Title	Component	Methods of production and extraction	Benefit area	Target	Patent category
US5,834,427 [Han and Shin, 2001a]	Casein phosphopeptide, casein containing same and process for the preparation thereof	Purified novel β -casein H derived CPP with a novel AA sequence	Bovine milk hydrolysed with trypsin Extraction of β -casein H CPP by anion exchange, cation exchange, precipitation by ferric ions and gel filtration chromatography	Improved ability for solubilising minerals and absorption	Human nutrition for calcium absorption	S
US0096741A1 [Slattery and Leonora, 2003]	Enhanced mineral delivery	CPPs from β -casein complexed with minerals	CPPs with Xaa-Ser(P)-Ser(P)-Glu-Glu sequences produced by genetic recombination or organic synthesis; CPP complexed with minerals	Bone density improvement and oral formulas	Oral and bone health	O
US20030195150 A1 [Reynolds et al., 2009]	Antimicrobial peptides	Non-glycosylated antimicrobial peptides, less than 100 amino acids	Casein hydrolysed with trypsin or rennet Precipitation at pH 4.6, centrifugation and peak collection (semi-preparative HPLC)	Antimicrobial for oral hygiene	Oral hygiene	O
US5,015,628 [Reynolds, 1991]	Anticariogenic phosphopeptides	Phosphopeptides with caries and gingivitis inhibition properties Treatment of bone diseases and mineral malabsorption	Casein hydrolysed by trypsin Purification of CPPs with ion exchange chromatography followed by UF or selective precipitation of CPPs followed by dialysis	Food-grade remineralising and antimicrobial phosphopeptides	Oral hygiene	O
US0075675 A1 [Reynolds, 2008c]	Stabilised calcium phosphate complexes	CPP-ACP or CPP-ACFP complexes formed at pH between 5.0 and 7.0 Oral composition containing about 2% of CPP-ACP or CPP-ACFP	Tryptic digest of casein + ACP or ACFP Microfiltration (0.1- μ m filters) to remove salts and inactive peptides	Treatment or prevention of dental caries, tooth enamel remineralisation, prevention of dental calculus, dental hypersensitivity dental erosion and corrosion	Oral hygiene	O
US683688 B2 [Dixon and Kaminsky, 2005]	Stable oral compositions comprising casein phosphopeptide complexes and fluoride	Compositions with fluoride, CPP-ACP and calcium chelators	Method of maintaining fluoride levels in an oral care composition (decrease of fluoride concentration in the product by the use by date <20% compared to the fresh product) with a safe and effective amount of CPPs, fluoride and calcium chelator	Remineralisation and/or recrystallisation of enamel and dentin surfaces for the treatment of dental caries	Oral hygiene	O
US6,780,844 [Reynolds, 2004]	Calcium phosphopeptide complexes	Method for making complexes of ACP and ACFP stabilised by phosphopeptides	Casein hydrolysed with trypsin at pH 9.0 Mixing with Ca^{2+} , Pi and F at pH 9.0 Filtering and drying of the complexes formed	Anticariogenic, remineralisation of tooth surface and dietary supplements with increased calcium bioavailability	Oral hygiene	O
US0063922A1 [Reynolds and Tyas, 2005]	Dental restorative materials	Composition for restorative materials (CPP-ACP and CPP-ACFP complexes)	Composition for dental restorative materials (glass ionomer cement, composite material) containing CPP-ACP or CPP-ACFP	Treatment and prevention of oral caries	Oral hygiene	O
US6,448,374B1 [Reynolds, 2002]	Production of phosphopeptides from caseins	Method for preparation of anticariogenic phosphopeptides ranging from 10,000 to 20,000 Da	Casein hydrolysed with trypsin UF and DF	Anticariogenic phosphopeptides	Oral hygiene	O

US5,227,154 [Reynolds, 1993]	Phosphopeptides for the treatment of dental calculus	CPPs (zinc/phosphopeptide complexes or aggregates)	Method for inhibiting dental calculus using oral compositions containing CPP salts with phosphatase inhibitors Casein digest with proteolytic enzymes	Inhibition of dental calculus	Oral hygiene	O
US5,981,475 [Reynolds, 1999]	Treatment for sensitive teeth	Oral composition for treatment of dental hypersensitivity	Oral composition containing specific phosphoproteins and phosphopeptides derived from caseins	Desensitizing of teeth using CPPs	Oral hygiene	O
US0,193,557 [Reynolds, 2008a]	Dental mineralisation	Dental mineralisation with ACP or ACFP stabilised by CPPs	Method for mineralising dental surface or subsurface using a protein-disrupting agent (bleach, detergent, chaotropic agent or protease) application followed by the application of complexes of ACP or ACFP and CPPs	Mineralising a dental surface or subsurface	Oral hygiene	O
EP 2 343 314 A2 [Reynolds, 2011]	Calcium fluoride phosphopeptide complexes	Stable complexes of ACFP stabilised by phosphopeptides	Tryptic digest of casein Isoelectric precipitation (pH 4.6) and microfiltration to isolate CPPs Formation of CPP-ACFP complexes by addition of CaCl_2 , NaH_2PO_4 and NaF to the CPPs at pH >7	Anticariogenic complexes with potential applications as dietary supplements	Oral hygiene, supplements, pharmaceutical compositions	O
US0297203 [Tancrédil et al., 2010]	Impact of calcium phosphate complex on dental caries	Chewing gum and confectionary compositions to reduce dental caries	Chewing gum or confectionary containing at least 3% (w/w) CPP-ACP, acting as an anticaries agent CPP-ACP can be encapsulated to allow gradual release in center-filled gums or confectionaries	Slowing down the progression and enhancing the regression of dental caries; the composition may reduce caries formation by 16.9% compared to product free of CPP-ACP Increase in acid resistance >4% and increase in remineralisation (at least by 10%)	Oral hygiene	O
US0215593 A1 [Reynolds, 2010]	Ionic complexes	Methods of making superloaded phosphoprotein-stabilized ACP or ACFP complexes with anticariogenic properties with a calcium content higher than 30 mol/CPP mol	Blending CPP-ACP (or CPP-ACFP) with at least an equal amount of calcium phosphate (in the form of CaHPO_4) at pH <7	Anticariogenic complexes for remineralisation of dental surfaces/subsurfaces Method for treatment and/or prevention of dental caries, erosion, corrosion, hypersensitivity and dental calculus	Oral hygiene Dietary supplements	O

UF = Ultrafiltration; DF = diafiltration; NaCN = sodium caseinate; E = extraction methodology; O = oral hygiene; F = formulation; S = new sequences.

patents deal with the selective extraction of CPPs using a combination of membrane separation, namely ultrafiltration employing a range of molecular weight cut-off membranes. CPP aggregation with bivalent salts or disaggregation by acidification or calcium sequestrants allow targeted size alteration in order to separate CPP from the non-phosphorylated peptides present in the hydrolysates using ultrafiltration [Brule et al., 1982, 1985, 1989, 1991, 1993b, 1994b]. Other extraction techniques use ion-exchange chromatography [Lihme et al., 1998]. An extraction protocol based on crosslinking of CPPs to chitosan beads has also been described [Koide et al., 1994]. In addition, the formation of chitosan-CPPs nanocomplexes mainly involving electrostatic interactions has recently been described in a research paper by Hu et al. [2011]. Many of the patents are relatively similar and are based on modifications of the sequence of steps involved in the extraction protocol, namely, modification of the size of CPP aggregates at different stages of the extraction procedure.

One third of the patents have been targeted at oral hygiene applications as CPPs have been associated with the regression of dental caries, anticalculus and desensitizing properties at the tooth level. Additionally, the development of new formulations or the discovery of new CPP sequences has been addressed in these patents. The application of CPPs in different formats either as a means to increase the solubility of minerals in beverages [Naito et al., 1995] or in the formation of clusters with high calcium contents [Holt, 2006] have been described. The manufacture of a sterile pharmaceutical composition for the management of lactating livestock animals has also been outlined [Iscovich et al., 2011]. The discovery of novel CPP sequences has been addressed in two patents [Han and Shin, 2001a, b].

A Generally Recognised As Safe status has been attributed to CPPs by the Food and Drug Administration and other regulatory bodies for their use in foods and oral care products [Cochrane et al., 2010]. An evaluation of the cytotoxicity of CPP-ACP paste has been carried out in vitro using rat fibroblasts [Bussadori et al., 2010] where the cells were covered with round coverslips coated with CPP-ACP. The study concluded that there was a low cytotoxicity for CPP-ACP paste, with a slight decrease in cell viability (80–90% cell viability after 4-hour exposure to a CPP-ACP paste). Nevertheless, cell viability was above 70% after longer exposure periods (i.e., 7 days). As CPP-ACP was only used topically, this level of toxicity was considered acceptable. Therefore, CPP-ACP was classified as safe for use in topical applications for den-

tistry [Bussadori et al., 2010]. A wide range of CPP ingredients and CPP-containing products are currently available in the marketplace [Phelan et al., 2009] (table 3). These CPPs originate from the different bovine caseins. Most commercial products containing CPPs are found in the oral hygiene sector in the format of pastes, dental creams and chewing gums. The other commercial applications of CPPs are in the format of soft drinks or dairy foods (table 3).

In summary, CPP sequences have been well characterised. The formation of CPPs naturally occurs in vivo during gastrointestinal digestion. At a commercial level, CPPs are mainly produced by the action of hydrolytic enzymes (pancreatic activities) on bovine milk protein substrates. CPPs have Generally Recognised As Safe status and are commercially available in the marketplace in the format of ingredients, oral hygiene and food products. Many patents have been filed in relation to CPPs. These mostly describe extraction techniques of CPPs from milk protein hydrolysates, followed by new oral hygiene products, food and animal feed formulations and finally the discovery of new CPP sequences.

3. General Mechanisms of Action of CPPs in the Oral Cavity

Cariou lesions can be naturally repaired by remineralisation [Chen and Wang, 2010]. Remineralisation is defined as a restoration of lost dental apatite [Cross et al., 2006]. However, this remineralisation process is not always sufficient to repair lesions, as demineralisation and remineralisation are cyclical events [Lussi, 2009; Peters, 2010] and a disruption in the overall balance of both processes can be detrimental to tooth repair (i.e., when demineralisation occurs at a faster rate than remineralisation) [Walker et al., 2010; Hamba et al., 2011; Pradeep and Prasans, 2011]. Therefore, lesions need to be repaired using different strategies that favour remineralisation [Chen and Wang, 2010; Peters, 2010]. If the lesions are detected at an early stage, they can be reversed with a non-invasive procedure to avoid caries development [El-sayad et al., 2009; Peters, 2010]. To be considered as an efficient remineralising agent, a product has to increase remineralisation, decrease demineralisation and be retained on the enamel surface in order to display activity [White, 1995]. This must be achieved before the next acid challenge [Borges et al., 2011]. The basic requirements for remineralisation agents include the following [Pitts and Wefel, 2009; Zero, 2009; Borges et al., 2011]: (1) be safe for

Table 3. Some commercial ingredients and products containing CPPs

Product type	Manufacturer	Format	Application
<i>Ingredients</i>			
CE90CPP	DMV, International	ingredient	–
Lacprodan D1-2021	Arla Foods	ingredient	– improves oral hygiene and uptake of vitamins and minerals
Peptigen 110	MD Foods	ingredient	–
Capolac	Arla Foods	ingredient	– helps mineral absorption
CPPB and CPPC	Armor Proteins	ingredient	–
CPP-I, II and III	Meiji Seika	ingredient	–
Recaldent	Cadbury Adams	ingredient	–
<i>Food products</i>			
Tekkotsu Inryou	Suntory	soft drink	helps mineral absorption
Kotsu Calcium	Asahi	soft drink	helps mineral absorption
Meiji Milk Recaldent	Meiji	milk beverage	maintains strong teeth
<i>Oral hygiene products</i>			
Trident white sugar gum	Cadbury	gum (with Recaldent)	actively strengthens and rebuilds teeth with Recaldent
Recaldent mints	Cadbury	mints	–
MI Paste (Prospec™ MI Paste/ GC Tooth Mousse ^a)			helps remineralise and rejuvenate teeth and reduces dental erosion
MI Paste Plus (GC Tooth Mousse Plus ^a)	GC		
Topocal C-5 ^b	Nulite Systems International	dental cream	–
Phoscal ^b	Nulite Systems International	–	–

– = Details not available.

^a Commercial name outside of the USA, Canada and Japan. ^b No longer commercially available.

human use and display an effective bioactivity; (2) display rapid precipitation on partially demineralised teeth; (3) transform into a stable apatite resistant to subsequent attack by bacteria and other erosion agents; (4) display a beneficial action over fluoride; (5) be more efficient than the demineralisation that naturally occurs following saliva erosion; (6) be active both at the surface and the subsurface of the lesion, and have the ability to diffuse through the biofilm and into the lesion subsurface.

3.1. Role of Fluoride in the Prevention of Dental Caries and Erosion

Different agents have been used to prevent caries and improve remineralisation of the enamel. Amongst these, fluoride has been widely used to prevent caries and dental erosion. Fluoride can precipitate at the enamel subsurface in the form of calcium fluoride (CaF₂) or fluoroapatite [Ca₁₀(PO₄)₆F₂] and protect dental enamel from erosion

during abrasive brushing and demineralisation [ten Cate, 1999; Lussi, 2009; Reynolds, 2009]. Fluoroapatite crystals have been reported to be relatively insoluble, allowing them to protect the enamel from further demineralisation [Peters, 2010]. Fluoride is the most widely used and the most efficient agent to aid remineralisation and prevent demineralisation of teeth, making it the reference agent against which new remineralisation agents are compared [Pfarrer and Karlinsey, 2009]. Nevertheless, the concentrations of fluoride utilized in over-the-counter toothpastes (around 1,000 ppm fluoride) may not be sufficient. Indeed, White [1995] reported that most over-the-counter toothpastes and mouth rinses did not contain sufficient fluoride to significantly react with sound enamel. Additionally, Lussi [2009] noted that there has been an increase in dental erosion even though most toothpastes are fluorinated. It appears that on its own and at dosages commonly used in commercial dental hygiene products, fluoride is not sufficient to completely prevent the development of dental caries [Rao et al., 2009].

Hydroxyl groups from hydroxyapatite can be replaced by fluoride yielding fluoroapatite $[\text{Ca}_5(\text{PO}_4)_3\text{F}]$, which is more resistant to acid erosion [Ostrom et al., 1984]. A relatively large amount of calcium is required to form fluoroapatite (10 calcium to 2 fluoride ions), suggesting that calcium availability could be a limiting factor in the formation of fluoroapatite and more generally in enamel remineralisation [Cross et al., 2007b; Reynolds, 2009; Walsh, 2009]. Furthermore, Reynolds et al. [2008] demonstrated in an in situ experiment that the availability of calcium and phosphate ions could be a limiting factor in the formation of hydroxyapatite.

3.2. Remineralisation Properties of CPPs

The combination of fluoride with a source of bioavailable calcium as an adjunct has been proposed for the treatment of early stage caries [Cross et al., 2006; Reynolds, 2009; Peters, 2010]. Indeed, the incorporation of CPP-ACP in a toothpaste with 1,100 ppm fluoride resulted in higher remineralisation than when fluoride was used on its own [Reynolds et al., 2008]. The utilisation of calcium phosphate on its own or per se has not been successful in increasing remineralisation of the tooth surface, mainly due to the low solubility of these ions especially in the presence of fluoride [Azarpazhooh and Limeback, 2008; Reynolds, 2009]. This low solubility results in an inefficient attachment to the tooth surface [Reynolds, 2008b]. Unhydrolysed caseins can have anticariogenic properties [Rodrigues et al., 2011], however these effects are only seen at relatively high doses [Aimutis, 2004; Cross et al., 2006, 2007b]. An in vivo study has been conducted supplementing caseins in the diet of rats infected with cariogenic *Streptococcus sobrinus* and plaque-forming *Actinomyces viscosus* [Guggenheim et al., 1999]. The results of this study clearly demonstrated the anticariogenic and antiplaque effect of caseins. Nevertheless, on a weight basis, the dose required to observe an anticariogenic effect was reported to be at least 10 times more for casein than for CPPs [Reynolds, 1998; Cross et al., 2006; Azarpazhooh and Limeback, 2008; Moezizadeh and Moayedi, 2009; Gupta and Prakash, 2011]. CPPs are therefore attractive for their anticariogenic action and their potency as compared to their original milk protein substrate. The positive role of CPPs on the enhancement of calcium and phosphate solubility and their stabilisation at the tooth surface has therefore been exploited as a non-invasive procedure to prevent tooth demineralisation [Cochrane et al., 2010]. CPPs pro-

mote enamel remineralisation of carious lesions by maintaining a supersaturated state of calcium at the enamel surface. CPPs have a relatively low dissociation constant for calcium phosphate, i.e. of the order of several mM, which may be responsible for an increased bioavailability of calcium phosphate [Cochrane et al., 2010]. They also act as buffering agents, preventing a pH decrease in the oral cavity [Rahiotis et al., 2008], the saliva and plaque [Kitasako et al., 2010]. This buffering capacity helps prevent the dissolution of hydroxyapatite from the enamel. Cross et al. [2007a] described the two main roles of CPPs as being a stabilisation of calcium phosphate in solution to prevent precipitation and crystal growth and a biomineralisation action with crystal growth promoter properties at the tooth surface.

During in situ remineralisation experiments, Reynolds et al. [2003] demonstrated a positive role for CPPs in stabilising ACP and delivering soluble calcium and phosphate to the enamel surface. The remineralisation obtained with sugar-free chewing gums containing CPP-ACP was 2 times higher than that obtained with chewing gums with $\text{CaHPO}_4/\text{CO}_3$ or CaCO_3 . This was despite the fact that the amounts of calcium were 8–13 times lower in the CPP-ACP chewing gum. This demonstrates the higher efficacy of CPP-ACP to deliver calcium phosphate at the enamel surface for optimum remineralisation [Reynolds et al., 2003]. Additionally, enamel remineralised following CPP-ACP delivery is more acid-resistant than normal enamel, making it more stable in respect to further acid challenge [Iijima et al., 2004; Reynolds, 2008b]. CPP-ACP was also shown to remineralise the interior of the lesions, whereas fluoride products only remineralise lesion surfaces [Shen et al., 2011]. In addition to their remineralising properties, CPP-ACP can bind to dental plaque [Rose, 2000; Azarpazhooh and Limeback, 2008]. In this respect, the action of CPP-ACP is very similar to that reported for fluoride, i.e., inhibition of demineralisation, enhancement of remineralisation and inhibition of bacterial enzymes [Walsh, 2009].

3.3. Vehicles of CPP-ACP for Use in the Oral Cavity

Different strategies involving CPP-ACP have been proposed in order to restore minerals that have been lost during the erosion process. These include [Gupta and Prakash, 2011]:

- Direct oral hygiene route via the utilisation of topical gels [Reynolds, 1999, 2008b; Kumar et al., 2008; Elsayad et al., 2009; Rehder Neto et al., 2009], or mouth

Table 4. Summary of agents used for dental remineralisation [adapted from Walsh, 2009]

Active ingredient	Mechanism of action	Particle size, nm	Commercial ingredient/product
Fluoride	Inhibition of demineralisation Enhancement of remineralisation (deposition of aggregates of calcium fluoride at the tooth surface) Inhibition of bacterial enzymes	41	Most commercial toothpastes
Tricalcium phosphate	Slightly increases levels of calcium in the plaque and saliva Some issues with its bioavailability (formation of calcium phosphate and calcium fluoride complexes)	10–5,000	Cerasorb® Bio-Resorb®
Dicalcium phosphate dehydrate	Increases levels of free calcium in the plaque fluid	–	Used in combination with fluoride pastes
Calcium glass	Reduction in supragingival plaque Reduction in gingival bleeding Bioavailability of calcium and phosphate may be low	–	Novamin™
Calcium salts	Desensitization of sensitive cervical dentin Issue with unstabilised calcium and phosphate (formation of insoluble calcium phosphate precipitates)	–	Enamelon™
ACP	Hydrolyses at pH 7.4 to form octacalcium phosphate then surface apatite Desensitising effects Improvement of surface defects of surface enamel Not a remineralising agent on its own	–	–
CPP-ACP	Remineralises enamel subsurface lesions (product formed following remineralisation = hydroxyapatite) Suppresses demineralisation Increases levels of calcium in the dental plaque and inhibits fermentation Buffering action in the plaque by providing calcium and phosphate ions Binds <i>Streptococcus mutans</i> impairing their incorporation in the dental plaque Anticalculus action (prevents calcium precipitation) Causes the regression of WSL	1.5	Recaldent™ (GC Tooth Mousse, MI Paste)
CPP-ACFP	Similar effects as CPP-ACP with a synergistic effect of fluoride Greater remineralisation than CPP-ACP below pH 5.5 Product formed following remineralisation = fluoroapatite	2.1	Combination of GC Tooth Mousse or MI Paste with a fluoride toothpaste

rinses [Reynolds et al., 2003, 2008] containing remineralising agents.

- Adjuncts for oral hygiene using other vehicles for CPP-ACP delivery such as chewing gum [Shen et al., 2001; Reynolds et al., 2003] and lozenges [Cai et al., 2003]. Sugar-free sorbitol- or xylitol-based chewing gums are being the most commonly tested vehicles for the application of CPP-ACP in human oral environment [Yengopal and Mikenautsch, 2009].
- Foods: dairy foods and especially cheese is known as a good source of minerals. Dairy products including milk supplemented with CPP-ACP [Walker et al., 2006, 2009] and yoghurt supernatants [Ferrazzano et

al., 2008] have been employed to deliver minerals to the tooth surface. Other vehicles such as confectionaries [Walker et al., 2010] and soft drinks [Manton et al., 2010] have also been described.

Different commercial products enriched in calcium phosphate are available on the market for the remineralisation of tooth surface (table 4): these include Enamelon™, Novamin™ and Recaldent™ [Azarpazhooh and Limeback, 2008; Reynolds, 2008b]. Enamelon and Novamin are, respectively, composed of amorphous calcium phosphate and a bioactive glass of calcium sodium phosphosilicate [Azarpazhooh and Limeback, 2008; Reynolds, 2008b; Rehder Neto et al., 2009]. Recaldent is

a stable and highly soluble CPP complex loaded with calcium, phosphate and hydroxide ions [Cross et al., 2007b]. CPPs can bind calcium via their phosphate residues, resulting in the formation of CPP-ACP nanoclusters with a radius about 1.5 nm [Reynolds, 2009]. These nanoclusters prevent nucleation and precipitation and increase calcium phosphate solubility [Reynolds, 1998; Rose, 2000; Azarpazhooh and Limeback, 2008]. An increase in calcium (6.5 times) and phosphate (7.9 times) concentration in saliva following utilisation of a paste containing Recaldent compared to a placebo product has been reported [Shen et al., 2011]. Recaldent carries more calcium, phosphate and hydroxide ions in comparison to open CPP-ACP complexes; therefore, it has been reported to be a more efficient anticariogenic agent [Cross et al., 2007b]. It has also been described as being less bitter and having a lower allergenic potential when compared to casein [Rodrigues et al., 2011]. Recaldent contains bovine milk-derived CPPs [with the sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu stabilising nanoclusters of ACP in metastable solution] [Shen et al., 2001; Cross et al., 2007b; Walsh, 2009] and is used in products such as Trident white sugar gum, Recaldent sugar-free gum, Recaldent mints™ and MI Paste (Tooth Mousse) [Azarpazhooh and Limeback, 2008; Gupta and Prakash, 2011] (table 3). The CPP composition of Recaldent has been characterised and it is mainly composed of α_{s1} -casein f(59–79)5P, β -casein f(1–25)4P, α_{s2} -casein f(46–70)4P and α_{s2} -casein f(1–21)4P [Cross et al., 2007a; Reynolds, 2008c]. Under alkaline conditions (pH 7–9), α_{s1} -casein f(59–79) and β -casein f(1–25), can bind a maximum of 21 and 24 Ca and 14 and 16 Pi, respectively [Cross et al., 2007a].

In summary, the natural repair of carious lesions in the oral cavity can be improved by supplementation with different compounds improving tooth remineralisation. These include the utilisation of remineralising agents such as fluoride, the reference agent for tooth remineralisation. The formulation of most over-the-counter toothpaste may not include sufficient amounts of fluoride for optimum remineralisation of tooth lesions. Combinations of fluoride and CPP-ACP as a source of bioavailable calcium have been successfully utilised to enhance remineralisation of teeth. CPP-ACP has been used in the formulation of oral hygiene products, as adjuncts for the oral hygiene and as food products. Owing to their low calcium and phosphate dissociation constants, their ability to stabilise calcium phosphate into solution and to prevent its early precipitation, CPPs display their remineralising properties allowing crystal growth at the tooth surface.

4. Methodology to Assess Tooth Remineralisation Properties of CPPs

A large number of studies have been conducted using different vehicles to assess remineralisation of enamel or dentin. From a methodology viewpoint, artificial lesion approaches have been extensively used to study caries as they show the same histological features as in natural caries and are easier to reproduce, making them highly suitable as a model to study remineralisation of enamel lesions [Kumar et al., 2008]. Single section lesions are sections of natural lesions [White, 1995], their utilisation allows accurate measurement of mineral changes within the lesions [White, 1995; Itthagarun et al., 2005]. The plaque formed in enamel sections is similar to the natural plaque from a microbiological and biochemical point of view [Itthagarun et al., 2005]. When in situ studies are used as a prediction tool for subsequent in vivo human intervention trials, this allows a better control for product-unrelated variables [ten Cate, 1999]. The lesions can be studied both with human and bovine teeth. Bovine teeth are a good model as they have been reported to be similar to human teeth in terms of physical and compositional characteristics [Wu et al., 2010; Wegehaupt et al., 2011]. Bovine teeth are easier to obtain than human teeth and can be sourced from the same geographical area, reducing inter-variability that can be caused by environmental and nutritional factors [Wegehaupt and Attin, 2010]. Additionally, they are normally not in contact with fluorides and do not present caries [Wegehaupt and Attin, 2010]. Furthermore, bovine incisors present a larger surface area than human teeth allowing the preparation of more than one sample per tooth, which can help minimise intertooth differences [Wegehaupt and Attin, 2010].

Differences between protocols to measure remineralisation and demineralisation generally come from the substrate used to conduct the experiment. Remineralisation experiments are generally carried out on lesions, whereas demineralisation protocols use a larger range of options including teeth that have been pretreated by remineralising agents or natural enamel or teeth with surface lesions [White, 1995]. Remineralisation models in vitro have been classified into three categories as follows: the pH-lattice ion drift method, which consists of exposing the tooth surface to a remineralising solution of constant volume that is supersaturated with remineralising agents. The pH and concentration of the remineralising agents are not regulated in this case and therefore are subject to changes over the time course of the experiment.

The second model is a flow-through technique to the tooth surface; the remineralising solution is supersaturated and has a large volume to ensure a constant thermodynamic driving force during remineralisation. The third model or titration-controlled composition procedure consists of constantly adding the remineralising agent in the remineralising solution [White, 1995]. Some *in vitro* protocols are classified as pH cycling; they consist of exposing the teeth to cycles of demineralisation and remineralisation. These protocols aim to reproduce the natural caries process while simulating changes in mineral saturation and pH that occur when subjects have plaque and consume carbohydrates at regular intervals [ten Cate, 1999].

In situ protocols are quite similar to *in vitro* protocols regarding the methodologies used to assess remineralisation and demineralisation of tooth surfaces. Studies carried out *in situ* utilise removable intra-oral appliances holding enamel slabs which previously went through a demineralisation protocol [ten Cate, 1999]. The demineralisation protocol can lead to the formation of subsurface lesions of 80–110 μm in depth [Azarpazhooh and Limeback, 2008]. Appliances can be worn by human subjects for a given period of time when the anticariogenic protocol is applied. They are then removed and the enamel slabs are analysed with different analytical techniques such as microradiography to study remineralisation of the enamel subsurface, the depth of the lesions, etc., and densitometric analysis to monitor changes in mineral content [Reynolds, 1997a; Azarpazhooh and Limeback, 2008]. More recently, non-destructive methods utilising *in situ* laser autofluorescence spectroscopy [Elsayad et al., 2009] and *in vitro* micro-computed tomography [Hamba et al., 2011] have been developed to study the remineralising potential of CPP-ACPs.

A very large number of studies have been carried out to assess the efficacy of CPP-ACP to remineralise tooth surface using *in vitro* and mainly *in situ* methodologies [Rodrigues et al., 2011]. At present the number of *in vivo* studies carried out is limited, but an increase in the number of human intervention trials reported in the literature is beginning to occur (table 5). The aim of these studies has been to assess the impact of CPP-ACP on the regression of dental caries and plaque [Reynolds et al., 2003; Morgan et al., 2008; Reynolds et al., 2008; Caruana et al., 2009] or on the regression of white spot lesions (WSL) which may appear following the use of intra-oral appliances [Andersson et al., 2007; Bailey et al., 2009; Beerens et al., 2010; Kitasako et al., 2010; Brochner et al., 2011].

5. Determination of the Role of CPP-ACP on Tooth Remineralisation and Prevention of Demineralisation *in vitro* and *in situ*

The maintenance of a target mineral concentration for healthy teeth is conditioned by the balance between demineralisation and remineralisation of the enamel. Most research on the action of CPPs in the mouth has been carried out with *in vitro* and *in situ* protocols employing a wide range of vehicles ranging from oral hygiene products (topical gels and mouthwashes) to adjuncts for oral hygiene (gums, lozenges) or foods (milk, yoghurt, sweets, soft drinks, etc.) [Gupta and Prakash, 2011].

5.1. CPP-ACP Action during Oral Hygiene Protocols

5.1.1. Remineralisation Potential of CPP-ACP on Carious Lesions

CPP-ACP has been applied in paste format *in vitro* to the enamel and dentin of bovine teeth that were previously subjected to demineralisation with a 0.1 M lactic acid buffer solution. The use of CPP-ACP resulted in an increase in the remineralisation of the dental enamel and dentin [Reynolds et al., 1999] as observed microscopically (field emission-scanning electron microscopy, FE-SEM). Similar results were obtained *in vitro* with primary human teeth (6-year-old lower incisors), showing higher remineralisation of enamel lesions with CPP-ACP compared to a 500 ppm NaF solution [Zhang et al., 2011]. This study [Zhang et al., 2011] was designed to address early childhood caries and concluded that there was a positive effect of CPP-ACP on early enamel lesions of primary teeth. The observed suppression of demineralisation and enhancement of remineralisation of tooth surfaces in the presence of CPP-ACP have been explained by a positive effect of CPP via the localisation of the ACP on the tooth surface. CPP-ACP is thought to prevent demineralisation and enhance remineralisation by increasing the concentration of calcium and phosphate ions, leading to a state of supersaturation [Reynolds et al., 1999].

CPP-ACP has been used to combat demineralisation during orthodontic treatment. The utilisation of fixed orthodontic appliances can often result in the accumulation of dental plaque and demineralisation around the brackets [Sudjalim et al., 2006; Andersson et al., 2007; Bailey et al., 2009; Xiaojun et al., 2009; Beerens et al., 2010; Peters, 2010]. As a consequence, the prevalence of demineralisation is higher with people wearing appliances as compared to people without orthodontic treatment

Table 5. Summary of human (in vivo) clinical studies assessing the remineralisation potential of CPP-ACP

Product tested	Participants, n	Duration	Results	Reference
<i>Test group:</i> mouthrinse with (1) 2% CPP-ACP Recaldent, (2) 6% CPP-ACP Recaldent, (3) unstabilised slurry of calcium and sodium phosphate <i>Control group:</i> deionised water	30 adults	5 days	Increase in Ca and Pi level of in the plaque in a dose-dependent manner; CPP-ACP localised at the bacterial surface and in the intercellular plaque matrix	Reynolds et al. [2003]
<i>Test group:</i> sugar-free chewing gum Recaldent pellet gum containing 9.5 mg of CPP-ACP <i>Control group:</i> none	30 adults	4 days	Increase in CPP level in the plaque; 132 ng of CPP/mg plaque (25% of this amount still present in the plaque 3 h after gum chewing)	Reynolds et al. [2003]
<i>Test group:</i> 3 months' treatment with CPP-ACP paste without fluoride (Topacal C5) + 3 months fluorinated toothpaste <i>Control group:</i> 6 months 0.05% NaF mouthwash + fluoride toothpaste (1,000–1,100 ppm)	26 adolescents	6 months (12 months follow-up)	63% WSL sites totally disappear with the CPP-ACP treatment vs. 25% for the control after 12 months (55 and 18%, respectively, after 6 months)	Andersson et al. [2007]
<i>Test group:</i> microabrasion of WSL followed by CPP-ACP paste (Tooth Mousse) application (15 min, twice daily) <i>Control group:</i> none	not disclosed	up to several months (not specified)	Natural tooth appearance recovered with elimination of superficial WSL	Ardu et al. [2007]
<i>Test group:</i> (1) mouth rinse (Recaldent) with 2% CPP-ACP + 450 ppm fluoride and (2) mouth rinse with 450 ppm fluoride <i>Control group:</i> deionised water	14 adults	5 days	Plaque fluoride level doubled with the fluoride mouth rinse as compared to the control Plaque fluoride level with the 2% CPP-ACP + fluoride mouth rinse more than two times that obtained with the fluoride mouth rinse	Reynolds et al. [2008]
<i>Test group:</i> sugar-free gum containing 54 mg CPP-ACP <i>Control group:</i> sorbitol-based sugar-free gum	2,720 adolescents	24 months	18% reduction in approximal caries and 53% greater regression with the CPP-ACP group as compared to the control group	Morgan et al. [2008]
<i>Test group:</i> Tooth cream with 10% CPP-ACP (Tooth Mousse/MI Paste) + fluoride toothpaste (1,100 ppm) and mouth rinse (900 ppm) <i>Control group:</i> placebo cream + fluoride toothpaste (1,100 ppm) and mouth rinse (900 ppm)	45 adolescents (post orthodontic population)	12 weeks	31% more regression of the WSL as compared to the control	Bailey et al. [2009]
<i>Test groups:</i> Tooth Mousse topical application after tooth brushing <i>Control group:</i> none	10 orthodontic patients (average age 17.7 years)	2 months' follow-up	Reduction of enamel demineralisation of WSEL	Zhou et al. [2009]
<i>Test groups:</i> (1) toothpaste with 2% CPP and (2) toothpaste with 1,190 ppm fluoride and 0.76% SMFP <i>Control group:</i> placebo toothpaste without CPP	150 adolescents	24 months	Regression of caries as compared to the control group No significant differences of decayed surface of the carious lesions in the CPP group and the SMFP group	Rao et al. [2009]
<i>Test group:</i> Tooth Mousse in combination with fluoride toothpaste <i>Control group:</i> fluoride toothpaste	60 adolescents	4 weeks	No significant difference in the regression of WSL between the test and the control groups	Brochner et al. [2011]
<i>Test group:</i> CPP-ACP (10% (w/w) – MI Paste) + non-fluoride toothpaste <i>Control group:</i> none	8 adults	24 months	Increase of the pH of WSEL during 24 months (from 5.94 to 6.70) toward that of sound enamel Visual improvement of the appearance of WSEL	Kitasako et al. [2010]
<i>Test group:</i> CPP-ACP [0.2% (w/w)] paste (Recaldent GC Tooth Mousse) applied with fluoride trays without rinsing <i>Control group:</i> healthy premolar of the same subjects studied	30 children (6–9 years) with molar incisor hypomineralisation	3 years 4 months' follow-up	More geometric, mature and mineralised MIH Increase in the potential MIH enamel structure	Baroni and Marchionni [2011]
<i>Test group:</i> CPP-ACP (Tooth Mousse) + sodium fluoride (1,450 ppm F) toothpaste <i>Control group:</i> sodium fluoride (1,450 ppm F) toothpaste	26 adults (22–31 years)	3 weeks	Significant reduction of laser fluorescence in the CPP-ACP group after 15 days, compared to the control group Reduction of the enamel surface porosity in the CPP-ACP group No difference between the 2 groups while performing a visual analysis of the fissures	Altenburger et al. [2010]

Table 5 (continued)

Product tested	Participants, n	Duration	Results	Reference
<i>Test group:</i> CPP-ACP (Tooth Mousse) + sodium fluoride topical gel <i>Control group:</i> no agent applied on the tooth surface	21 adolescents (13–17 years)	60 days	Prevention of demineralisation of the enamel around orthodontic brackets with CPP-ACP and fluoride; no significant difference between CPP-ACP and fluoride	Uysal et al. [2010]
<i>Test group:</i> CPP-ACP [0.2% (w/w)] + sodium fluoride (900 ppm) paste (MI Paste Plus) in combination with fluoride toothpaste <i>Control group:</i> fluoride-free control paste (Ultradent) in combination with fluoride toothpaste	54 orthodontic adolescents after debonding	12 weeks (follow-up 3 months)	No significant change in the size of the WSL between the 2 groups Decrease in the percentage of aciduric bacteria from 47.4 to 38.1% and <i>S. mutans</i> from 9.6 to 6.6% No advantage of CPP-ACFP paste in addition to normal oral hygiene	Beerens et al. [2010]
<i>Test group:</i> CPP-ACP (MI Paste) applied after tooth brushing with a fluoride paste <i>Control group:</i> placebo fluoride paste	60 orthodontic adolescents	3 months	Reduction of enamel decalcification index score by 53.5% with MI Paste Reduction of the number of WSL and protective effect of CPP-ACFP	Robertson et al. [2011]
<i>Test group:</i> CPP-ACP (Tooth Mousse) on demineralised teeth <i>Control group:</i> placebo gel on demineralised teeth/sound and demineralised teeth with no topical treatment	40 adolescents (10–16 years)	1 month	Formation of an amorphous layer on demineralised teeth following CPP-ACP treatment	Ferrazzano et al. [2011]

SMFP = Sodium monofluorophosphate; WSEL = white spot enamel lesions.

[Sudjalim et al., 2006; Beerens et al., 2010; Wu et al., 2010]. This can cause the appearance of WSL at the tooth surface that can further develop into caries [Sudjalim et al., 2006; Beerens et al., 2010; Uysal et al., 2010]. WSL development has been associated with inadequate hygiene and with plaque build-up in areas that are difficult to access during cleaning procedures or natural self-cleansing mechanisms such as oral musculature and saliva movement [Sudjalim et al., 2006]. Recently, the secondary treatments to prevent WSL and to improve their appearance after debonding have been reviewed by Bergstrand and Twetman [2011]. Bovine enamels were treated with Tooth Mousse and enamel remineralisation was assessed employing polarised digital imagery [Wu et al., 2010]. Most studies to assess remineralisation of dental enamel have been carried out with macroscopic methods, clinical examination and quantitative light-induced fluorescence, but a restricted number of studies utilised polarised digital images. Polarised filters avoid reflections coming from the flash in digital photography, these reflections can cause an overestimation of the actual WSL at the tooth surface [Wu et al., 2010]. The advantage of circular polarised lens over linear polarised lens is that it gives a clearer image of the actual demineralised areas [Wu et al., 2010]. The application of Tooth Mousse resulted in a reduction in the demineralised areas and this

effect was more pronounced when CPP-ACP was combined with fluoride toothpaste.

Synergistic effects of CPP-ACP and fluoride have been reported in several studies [Kumar et al., 2008; Reynolds, 2008b; Shen et al., 2011]. When CPP-ACP (Tooth Mousse) was used as a topical coating following treatment with fluorinated toothpaste, the reduction of lesions was of the order of 13.1% (in comparison to 7.0% for the fluorinated toothpaste alone and 10.1% for the CPP-ACP paste) [Kumar et al., 2008]. This result suggests that a combination of fluoride and CPP-ACP can help to improve the appearance of enamel lesions. Fluoride is important at plaque level as it is involved in the formation of fluoroapatite and can therefore promote remineralisation of the enamel subsurface [ten Cate, 1999; Walsh, 2009]. There may be some incompatibility issues between fluoride and CPP-ACP. In formulations containing the two substances, the availability of fluoride can decrease [Pfarrer and Karlinsey, 2009]. A practical solution to this issue includes physical separation with the use of a dual chamber packaging, formulation changes to minimise interactions between fluoride and CPP-ACP or the reduction of water content [Pfarrer and Karlinsey, 2009]. Nevertheless, it was reported that in a product such as MI Paste, ACP should not interact with fluoride, and precipitate as CaF, due to the relatively low fluoride concentration (900 ppm) [Gupta

and Prakash, 2011]. When a fluoride mouth rinse was supplemented with 2% CPP-ACP, the incorporation of fluoride in the plaque was shown to increase by a factor of more than 2 [Reynolds et al., 2008] in an in situ experiment. Additionally, 2% CPP-ACP gave higher remineralisation with and without acid challenge than a 2,800 ppm fluoride toothpaste. The 2% CPP-ACP toothpaste also gave higher remineralisation results than the 1,100 ppm fluoride paste [Reynolds et al., 2008]. The combination of Tooth Mousse Plus (containing 900 ppm fluoride) [Walsh, 2009] and the oral mouthwash Relief ACP containing fluoride was studied using a non-destructive optical method based on laser autofluorescence spectroscopy to monitor lesion development/resorption [Elsayad et al., 2009]. A synergistic effect of fluoride and CPP-ACP was demonstrated. Remineralisation following utilisation of both products resulted in superimposed spectra for the sound enamel subsurface and the remineralised subsurface. When GC Tooth Mousse was used on its own, a shift from 540 (sound enamel) to 500 nm (remineralised enamel) for the peak maximum was seen. Similarly, Shen et al. [2011] reported a synergy between CPP-ACP and fluoride with a 3.7-fold increase in remineralisation level following the use of Tooth Mousse Plus as compared to a 1,000 ppm fluoride toothpaste. Remineralisation of tooth specimens was shown to be higher with CPP-ACFP when compared to CPP-ACP [Jayarajan et al., 2011]. The use of CPP-ACFP was advised over fluoride as a slow delivery system for fluoride in the lesions. This gradual release allows curing of the lesions as opposed to high fluoride concentrations, which result in hypersurface mineralisation, preventing repair of the lesion [Jayarajan et al., 2011]. The localisation of the ACFP was reported to increase the degree of saturation with respect to fluoroapatite [Cross et al., 2006]. CPP-ACFP complexes possibly prevent rapid precipitation of fluoroapatite at the tooth surface, allowing a gradual release of calcium, phosphate and fluoride ions [Cross et al., 2007b]. All three ions (fluoride, calcium and phosphate) were free to diffuse at the enamel subsurface [Reynolds, 2009]. An estimated diffusion coefficient of $3 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}$ for remineralisation has been reported [Reynolds, 1998; Cross et al., 2006].

Contradictory results have been reported regarding the efficacy of toothpastes containing CPP-ACP. Rehder Neto et al. [2009] compared the effect of different oral pastes on bovine dental surfaces with induced caries-like lesions (pH cycling protocol). Remineralisation of the tooth surfaces was seen with the different remineralising agents tested (toothpaste with fluoride, MI paste, MI paste with fluoride and Tooth Revitalising Paste contain-

ing Novamin). Higher amounts of mineral changes on the enamel surfaces were obtained with the fluoride toothpaste and Tooth Revitalising Paste (7.2 and 6.7%, respectively). MI paste and MI paste with fluoride gave lower changes in mineral levels of 3.3 and 3.2%, respectively [Rehder Neto et al., 2009]. The pastes containing the CPP-ACP were effective in increasing remineralisation, nevertheless, higher remineralisation was achieved with fluoride and Novamin pastes. In another study, the microhardness of teeth was investigated. CPP-ACP paste and a fluoride toothpaste allowed remineralisation around an orthodontic bracket, but in contrast to a previous study [Rehder Neto et al., 2009], no significant difference could be measured between CPP-ACP and fluoride [Uysal et al., 2010]. Additionally, the in vitro study conducted by Lata et al. [2010] did not demonstrate any benefit on the remineralisation of subsurface lesions while using CPP-ACP on its own or when applied in a fluoride varnish. In this study, the remineralisation of enamel was only seen at the surface level with both treatments (CPP-ACP alone and CPP-ACP applied in a fluoride varnish) [Lata et al., 2010].

5.1.2. Protective Effect of CPP-ACP during Erosion

In in vitro studies, Tooth Mousse was shown to be effective in reducing erosion of dentin in conditions involving heavy attrition (physical wear) against opposing enamel surfaces [Ranjitkar et al., 2009a]. Erosive wear of dentin and enamel was reduced following the application of Tooth Mousse after an in vitro simulation of dentin and enamel wear following toothbrush abrasion [Ranjitkar et al., 2009b]. It was demonstrated that four topical applications of CPP-ACP paste (ProspecTM, MI Paste) were effective in preventing dental erosion [Tantbirojn et al., 2008]. After contact with a cola beverage, treated teeth presented a harder enamel surface compared to untreated teeth [Tantbirojn et al., 2008]. These results are in agreement with another study which tested the effect of CPP-ACP on the microhardness of enamel following erosion with a cola drink, where CPP-ACP treatment resulted in a 13% increase in tooth hardness [Panisch and Poolthong, 2009]. Intermittent application of Tooth Mousse reduced dentin wear, and continuous application resulted in a diminution of dentin wear rate [Panisch and Poolthong, 2009]. Similar results were obtained in vitro with white wine (White Riesling, pH 3.5) erosion of human teeth, where the application of Tooth Mousse resulted in reduction of the erosion depth both for enamel and dentin [Piekarz et al., 2008]. Tooth Mousse has been used in vitro to remineralise a tooth specimen following an erosion protocol with chlorinated water [Vongsawan et al., 2010].

The microhardness of the teeth was restored following application of Tooth Mousse or the immersion of the teeth in a commercial high-calcium milk (Anlene™). This study demonstrated the remineralisation potential of CPP and high-calcium milk following tooth erosion with chlorinated water [Vongsawan et al., 2010]. An in situ study conducted on the remineralisation potential of CPP-ACP after erosion with Coca Cola also showed the synergistic effect of fluoride and CPP-ACP [Srinivasan et al., 2010] where remineralisation increased from 46.2 (Tooth Mousse) to 64.25% (Tooth Mousse Plus containing 900 ppm fluoride). The synergistic effect of CPP-ACP and fluoride has been attributed to the formation of a stabilised ACP phase [Kumar et al., 2008; Reynolds, 2008b; Moezizadeh and Moayedi, 2009].

In contrast, different studies showed no protective effect of CPP-ACP on erosion. Recently, Wegehaupt et al. [2011] conducted an in situ study where they evaluated the protective effect of CPP-ACP following erosion of bovine teeth with a soft drink (Sprite light). The baseline surface microhardness of the teeth could not be restored both when Tooth Mousse or fluoride mouth rinse were used following the erosive protocol. When CPP-ACP was applied to bovine enamel in vitro, no significant decrease in the reduction of abrasion (tooth brushing)/erosion (demineralisation with HCl) could be demonstrated [Wegehaupt and Attin, 2010]. These results suggest that there was no positive effect of CPP-ACP on the reduction of tooth wear [Wegehaupt and Attin, 2010]. Nevertheless, in this study, the CPP-ACP mousse was applied only once a day as opposed to other studies where application could vary from continuous to several daily intermittent applications of Tooth Mousse [Ranjitkar et al., 2009a, b]. This observation raises the question of the frequency/duration of CPP-ACP application required to achieve a protective effect on dental abrasion and erosion. Similar results were found where the application of CPP-ACP on human teeth in vitro did not improve surface nanohardness [Wang et al., 2011]. This study was carried out with clinically relevant parameters with an acid challenge applied for 3 min with orange juice and an application time onto the tooth surface of 3 min for the CPP-ACP paste. No protective effect on erosion could be seen using SEM as CPP-ACP failed to form a continuous layer at the tooth surface and instead was deposited as scattered granules. The difference with other studies which found a protective effect of CPP-ACP was attributed to the shorter application time used in this study. Furthermore, at the low pH of the orange juice (pH 3.6), CPP net charge is positive, reducing its affinity for the enamel, which may explain the results obtained [Wang et

al., 2011]. In contrast, a study carried out in vitro with a similar application time of 3 min found a protective effect of CPP-ACP on dental erosion of human teeth with an acid challenge (Cola, pH 2.44) [Poggio et al., 2009]. Structural changes were observed on tooth surfaces using atomic force microscopy, with tooth cavity being more filled when CPP-ACP was used after the acid challenge as compared to no CPP-ACP application. Erosion cavity depth was reduced from 50 (no CPP-ACP application) to 20 µm (CPP-ACP application), showing the protective effect of CPP-ACP. These results contradict the findings of Wang et al. [2011] regarding pH and time exposure on the protective effect of CPP-ACP. The main difference between both studies may arise from the rinsing procedure of the teeth after application of the CPP-ACP paste; a relatively harsh rinsing procedure (50 s under tap water followed by 10 s with deionised water and 5 s drying with oil-free air) was applied in the study by Wang et al. [2011]. The teeth were rinsed with deionised water in the study by Poggio et al. [2009]. The rinsing procedure could affect the efficiency of the active ingredient as it may act for longer if not fully rinsed off the teeth. For this reason it has been proposed not to vigorously rinse teeth after the utilisation of fluoride-containing toothpastes on tooth brushing. Rinsing with a small amount of water [Rodrigues et al., 2011] and even a no-rinse procedure [ten Cate, 1999; Peters, 2010] has been advised in order to increase retention of the remineralising agent (fluoride) in the mouth.

Other studies concluded that no synergy occurred between CPP-ACP and fluoride; for instance during the early stages of erosion caused by a cola beverage on the enamel subsurface of bovine teeth [Tantbirojn et al., 2008]. The level of fluoride used in the study was nevertheless relatively low: 1 ppm fluoride in artificial saliva as opposed to several hundred parts per million fluoride in previous studies showing synergistic effects between CPP-ACP and fluoride. Similarly, in the study by Wang et al. [2011], no additional protection of CPP-ACP in the presence of 900 ppm of fluoride could be observed. This study concluded that there was no protective effect of protective agents (CPP-ACP, CPP-ACFP or Novamin) on erosion caused by an acid challenge.

5.2. Role of CPP-ACP in vitro and in situ during the Use of Adjuncts for Oral Hygiene: Lozenges and Chewing Gums

Lozenges and chewing gums are classified as adjuncts for oral hygiene that can be used in addition to the con-

ventional tooth cleaning procedures. The advantage of using chewing gum as a carrier is that it has a longer residence time in the mouth as compared to toothpaste or mouthwash [Sanares et al., 2009]. Remineralising agents that are retained in the oral cavity are more efficient as they can act for longer at the tooth surface. Chewing gum additionally acts on increasing saliva flow, which has been positively associated with an increased protection of the teeth [Imfeld, 1999]. The importance of controlled release of anticaries agents in the oral cavity has been stressed in different review papers [Pitts and Wefel, 2009; Chen and Wang, 2010].

Addition of CPP-ACP in lozenges was shown to induce an increase in the remineralisation of subsurface enamel lesions by 78 and 176% when the amount of CPP-ACP was increased from 18.8 to 56.4 mg, respectively [Cai et al., 2003]. Similar results were obtained regarding the incorporation of CPP-ACP in sugar-free gums (Recaldent) with a 102 and 152% remineralisation increase with 18.8 to 56.4 mg CPP-ACP, respectively [Shen et al., 2001]. A larger range of CPP-ACP amounts was tested with sugar-free gums (0.19, 10, 18.8 and 56.4 mg) and the percentage of remineralisation of the enamel showed a dose-response pattern between CPP-ACP dose and remineralisation of the enamel subsurface [Shen et al., 2001].

Trident White™ gum containing CPP-ACP was compared to two sugar-free gums without CPP-ACP and the results showed that this product allowed higher remineralisation than the non-supplemented sugar-free gums after 14 days' treatment. The remineralisation obtained with Trident White gum was 107% and this was 75% higher than with the regular chewing gums, Orbit and Orbit Professional, respectively [Manton et al., 2008]. Similar results were found with chewing gum containing urea where lesion depth in situ was reduced by 10.1% in the presence of 47 mg CPP-ACP [Itthagarun et al., 2005]. Urea can be degraded by bacteria in the plaque into ammonia, which raises plaque pH.

Another clinical study was carried out with sugar-free chewing gums containing CPP-ACP to remineralise lesions on human teeth mounted on oral appliances. This clinical trial investigated the acid resistance of enamel lesions remineralised in situ with sugar-free chewing gum containing 18.8 mg of CPP-ACP (Recaldent) [Iijima et al., 2004]. The remineralisation determined on the subsurface lesions of palatal appliances after 8 and 16 h of acid challenge was about 2 times higher with the Recaldent chewing gum as compared to a chewing gum without CPP-ACP. When the appliances were subjected to a second acid challenge, the remineralisation was similar both

with the CPP-ACP and the control chewing gums. The demineralisation was seen more underneath the remineralised zone, which indicated that the enamel remineralised with CPP-ACP was possibly more resistant to acid challenge than normal enamel. However, the composition of both enamels may differ, normal enamel has been reported to be calcium-deficient and made of carbonated apatite that is less resistant than hydroxyapatite [Iijima et al., 2004]. Contradictory results were found in a similar study that was conducted in situ with bovine enamel slabs with subsurface lesions. The human subjects chewed four gums daily over a period of 14 days [Schirrmeyer et al., 2007]. No benefit of CPP-ACP gums could be demonstrated in this study.

5.3. Influence of Foods Supplemented with CPP-ACP on Tooth Remineralisation

Milk and dairy products, especially cheese, naturally display an anticariogenic or cariostatic effect [Kashket and de Paola, 2002; Cross et al., 2006; Gupta and Prakash, 2011] which may be explained by the intrinsic presence of casein and calcium phosphate. Nevertheless, most of the calcium present in milk is bound to casein micelles; therefore, addition of CPP-ACP to milk has been proposed as a means to increase the bioavailability of calcium ions. Yoghurt supernatants obtained after centrifugation of yoghurt were used as a natural source of CPPs. Tooth specimens were subjected to demineralisation with lactic acid and then immersed for 96 h in yoghurt supernatants [Ferrazzano et al., 2008]. This resulted in an increase in enamel remineralisation as compared to control teeth that were not in contact with the yoghurt supernatants. Direct addition of CPP-ACP to milk has been attempted to achieve an enhanced remineralisation of the tooth subsurface in situ [Walker et al., 2006, 2009]. Three treatments were applied to subjects wearing removable appliances with attached enamel slabs containing subsurface lesions. The treatments consisted of a daily consumption of milk without CPP-ACP (treatment 1), milk with 0.2% CPP-ACP (treatment 2) and milk with 0.3% CPP-ACP (treatment 3) over 15 days [Walker et al., 2009]. In a previous study, the same approach was followed with a control that consisted of milk, and two other treatments with milk containing 0.2 and 0.5% (w/v) CPP-ACP [Walker et al., 2006]. In both studies, remineralisation of the slabs was observed for the three treatments. Nevertheless, higher remineralisation was obtained in the presence of CPP-ACP with an increase by 81 and 164% for treatments

2 and 3, respectively, as compared to treatment 1 [Walker et al., 2009]. The extent of remineralisation observed for the 0.2 and 0.5% CPP-ACP was reported to be 70 and 148% higher than for milk in the other study [Walker et al., 2006]. Milk naturally enhances remineralisation of enamel subsurface, which is in agreement with its anticariogenic properties. Addition of CPP-ACP to milk allows an increased remineralisation of the enamel surface in a dose-dependent manner. It has been proposed that owing to its intrinsic remineralisation properties, milk could be a better carrier for enamel remineralisation agents as compared to other vehicles [Walker et al., 2009].

Food products such as sugar confectionaries have been utilised in an in situ study as a vehicle for CPP-ACP [Walker et al., 2010]. The addition of CPP-ACP in the sugar confectionary resulted in an increase in the mineral content of both the surface and body of the tooth lesions with a dose-dependent effect on the remineralisation observed on the lesions. When CPP-ACP was added to sugar-free and sugar-containing confectionaries, differences were observed with the remineralisation being more effective with the sugar-free confection. In the presence of sugar, the pH of the enamel slabs decreased below pH 5.5, where remineralisation was less effective. Based on the above results, formulations of sugar confectionaries with CPP-ACP have been proposed as a means to decrease their cariogenicity [ten Cate, 1999].

The prevalence of dental erosion has been associated with an increase in the consumption of soft drinks. Therefore, CPP-ACP was formulated into acidic soft drinks in order to assess its influence on dental erosion. The addition of 0.2% (w/v) CPP-ACP to acidic soft drinks decreased erosion depth in comparison to soft drinks without CPP-ACP [Manton et al., 2010]. This result suggests that CPP-ACP could be added to soft drinks in order to reduce their erosive potential.

In summary, numerous studies have been conducted in vitro and in situ to assess the remineralising potential of CPP-ACP delivered in the format of oral pastes, adjuncts for the oral hygiene or foods. The outcome of most studies supports the positive role of CPP-ACP and CPP-ACFP as prophylactic agents for caries, erosion and WSL. Although some contradictory results are seen in the literature, differences in study outcomes seem to arise from the protocol set-up, notably, the exposure time of teeth to CPP-ACP appears to be a crucial parameter for bioactivity. The vehicle also plays an important role in the efficacy of CPP-ACP. From most studies carried out in vitro and in situ to date, there seems to be strong evidence for CPP bioactivity in the oral cavity.

6. Role of CPP-ACP in Combating the Formation of Biofilms

A wide range of bacteria is naturally found in the oral cavity in the mucosa and dental plaque [Pitts and Wefel, 2009; Marsch et al., 2011]. These bacteria are organised in a biofilm adhering onto the tooth surface [Marsch et al., 2011]. They are characterised by their ability to produce acid following the fermentation of dietary carbohydrates. This acidification is responsible for enamel demineralisation [Aimutis, 2004; Cross et al., 2007a; Hannig and Hannig, 2010]. The use of antibacterial agents has been proposed to control the development of dental plaque [Chen and Wang, 2010]. As already outlined, evidence on the positive role of CPP-ACP complexes against the formation of plaque has been well established [Rose, 2000; Rahiotis et al., 2008]. CPP-ACP mainly acts on the plaque through its buffering capacity, counteracting the pH reduction caused by bacterial growth at plaque level. The advantage of using CPPs as antimicrobial agents to combat the development of dental plaque is that they are natural agents and are not associated with the major drawbacks of antibiotics, i.e., the development of bacterial resistance to therapeutic agents and undifferentiated killing of bacteria [Chen and Wang, 2010].

6.1. The Buffering Capacity of CPP-ACP in Plaque

Caries risk is generally assessed by the measurement of pH in the dental plaque [Caruana et al., 2009]. CPP-ACP acts by binding to the hydroxyapatite and diffusing inside of the dental plaque where it exhibits a buffering capacity and increases calcium content [Chen and Wang, 2010]. The buffering capacity of CPP-ACP is useful in countering the pH reduction provoked by acidogenic bacteria at plaque level. This effect of CPP-ACP has been demonstrated following carbohydrate challenge in a short-term study conducted in vivo with human subjects utilising a toothpaste (GC Toothpaste) containing CPP-ACP [Caruana et al., 2009]. The impact of CPP-ACP after a carbohydrate challenge was studied using a micro-touch method based on a miniature pH electrode [Caruana et al., 2009]. The application of GC Toothpaste prior to the acid challenge reduced the decrease in plaque pH. Average plaque pH was 5.0 without the application of CPP-ACP paste versus pH 5.8 when CPP-ACP was used. In contrast, no conclusive effect of CPP-ACP was found after 3 weeks of utilisation by 25 patients wearing fixed orthodontic appliances [Marchisio et al., 2010]. The pro-

cedure used in this study was nevertheless unclear as details are missing notably regarding the way in which Tooth Mousse was applied on the teeth and regarding other procedures used for subject oral hygiene such as the utilisation of fluorinated toothpaste. Additionally, this clinical trial did not include a control group, which makes the interpretation of the results more difficult. The authors concluded that no clear short-term effect of CPP-ACP could be seen.

A protective effect of CPP on the disruption of hydroxyapatite crystals with acids was demonstrated in the absence of bacteria [Kanekanian et al., 2008]. This suggests that CPP may adsorb at the surface of hydroxyapatite via physicochemical interactions, forming a layer allowing CPPs to display their protective effect. Protection of hydroxyapatite was shown to follow a dose-dependent pattern and was maximal at a CPP concentration of 10 mg·ml⁻¹. No differences in terms of hydroxyapatite protection could be seen with CPPs saturated with calcium and with CPPs where 70% of the calcium was removed, suggesting that the protection of hydroxyapatite was not achieved through a buffering capacity of Ca but through that of CPPs [Kanekanian et al., 2008]. Nevertheless, calcium and phosphate ions have an indirect role on pH control at the enamel level, as the deposition of ions can reduce the diffusion of plaque acids in the enamel [Peters, 2010].

6.2. Interaction of CPP-ACP with Dental Plaque Biofilms

It has been demonstrated that CPP-ACP can directly bind the bacterial surface [Cross et al., 2006]. The binding of CPP-ACP at the bacterial surface may be specific as the binding was not the same depending on the bacterial strain. Binding of CPP-ACP to bacterial cells was established directly with the bacterial cell surface [Reynolds et al., 2003]. At the bacterial cell and pellicle surface, binding may involve hydrophobic interactions and hydrogen bonds [Reynolds et al., 2003]. Binding of CPP-ACP to the dental plaque resulted in an increase in the Ca and Pi levels when mouthrinse was utilised by human subjects, leading to a 118 and 57% increase in Ca and Pi, respectively. At the plaque level, CPP-ACP acts as a reservoir of soluble calcium phosphate ions that can diffuse into the enamel subsurface to promote remineralisation [Reynolds et al., 2003]. After incorporation in the dental plaque, CPPs can remain at relatively high levels. Three hours after the consumption of chewing gums containing CPP-

ACP, the level of CPPs in the plaque was 4.6-fold higher than in a control baseline plaque [Reynolds et al., 2003]. A half-life of 124.8 min has been reported for CPP in the plaque [Cochrane et al., 2010]. The time-dependent decrease in CPP concentration in plaque may be due to the action of enzymes from the dental plaque (peptidases and phosphatases) that can degrade CPPs [Reynolds et al., 2003].

CPP-ACP has been shown to reduce calcium diffusion in a streptococcal model plaque from *Streptococcus mutans* R9 isolated from human caries. This decrease in calcium diffusion allowed a potential reduction of calcium loss from the plaque and therefore increases the availability of calcium that could participate in remineralisation of the enamel surface [Rose, 2000]. An average of 3 Ca²⁺ per CPP-ACP unit can bind to bacterial cells, corresponding to a binding capacity of 0.16 g CPP-ACP/g wet wt cells at pH 7. This number depends on the pH: at pH 5, which mimics the acidic conditions prevailing during carious infections, the binding capacity decreases to 0.11 g/g wet wt cells due to a reduction in the overall charge of the CPP-ACP complex, reducing binding sites for CPP-ACP. This resulted in an increase in calcium diffusion in the plaque at pH 5 as compared to pH 7 [Rose, 2000].

Germanium (Ge) crystals mounted on orthodontic appliances with a custom-made retainer have been used to study the formation of biofilms after treatment of the crystals with Tooth Mousse (containing 10 wt% CPP-ACP complex) in situ [Rahiotis et al., 2008]. The orthodontic appliances were retained in the oral cavity of the subjects for different periods of time ranging from 30 min to 1 week. The formation of biofilms on the untreated Ge crystals was observed as early as 30 min, whereas biofilm formation with the CPP-ACP-treated Ge only started after 24 h. CPP-ACP complexes that adsorb at the enamel surface may cause an increase in the negative net charge, which act as repulsion forces for micro-organism attachment to the enamel surface. CPP-ACP crystals appeared at the surface of Ge-treated appliances, with crystal growth (nucleation and crystallisation) increasing over time [Rahiotis et al., 2008]. The conclusion from this work has to be carefully extrapolated to tooth surfaces. It has been pointed out by other authors [Hannig and Hannig, 2010] that in contrast to the tooth surface, Ge is not a biomineral (mineral naturally produced in living organisms) and may therefore react differently.

In summary, CPP-ACP may help combat dental plaque as it can directly bind to plaque bacterial surfaces and protect hydroxyapatite through its buffering capacity.

This binding may involve hydrophobic interactions and hydrogen bonds. CPP-ACP also reduces diffusion of calcium and phosphate ions in the plaque allowing longer residence in the plaque to remineralise carious lesions and also contribute to the buffering capacity. A long half-life time for CPP-ACP (124.8 min) has been demonstrated in dental plaque, which may be relevant for sustained bioactivity in the oral cavity over time.

7. Other Applications of CPP-ACP: Therapeutic Uses in Dental Treatment

Most of the applications of CPP-ACP described in the literature have targeted oral hygiene and more particularly the regression of dental caries and erosion via remineralisation and a decrease in demineralisation together with dental plaque regression.

7.1. Dentin Hypersensitivity

Other applications of CPP-ACP are found for treatment of dentin hypersensitivity. Successful application of CPP-ACP to reduce the pain experienced by subjects susceptible to dentin hypersensitivity has been reported [Kowalczyk et al., 2006]. Dentin hypersensitivity was reduced in an *in vivo* study carried out with 13 patients when a CPP-ACP paste was used as a topical treatment [Kowalczyk et al., 2006]. The elimination of pain could be seen in 28% of the teeth following the treatment. Nevertheless, this effect appeared to be limited in time as the sensation of pain was experienced again as early as 7 days after discontinuation of the application of CPP-ACP (with around 12% of the teeth showing no pain sensation). Despite its effect on dentin hypersensitivity, CPP-ACP was not as efficient as other agents already on the market [Kowalczyk et al., 2006]. In an *in vivo* study including 48 patients, hypersensitivity following scaling and root planning was also significantly reduced following the application of Tooth Mousse. The reduction of discomfort observed with Tooth Mousse was significantly higher than the control over a period of 10 days [Gugnani et al., 2008]. In another *in vivo* study, no benefit of CPP-ACP could be demonstrated. Following a whitening procedure, tooth sensitivity was significantly reduced with subjects using sugar-free gums within the 24 h of the treatment. Nevertheless, no significant difference was seen between the groups using sugar-free gums with or without CPP-ACP [Tang and Millar, 2010].

7.2. Avulsion Injury

CPP-ACP has also been successfully used in avulsion injuries, which is a traumatic dental injury characterised by complete displacement of the tooth from the alveolar socket, followed in some cases by an injury to the periodontal ligament (PDL) [Cehreli et al., 2008]. The avulsed teeth can be re-implanted; nevertheless, there is a need for a re-implantation medium to maintain viability of the PDL cells. Milk has been reported to support PDL cell viability, therefore, Cehreli et al. [2008] tested the efficacy of CPP-ACP present in Tooth Mousse as a re-implanting medium for PDL cells. They studied the effect of different dilutions of Tooth Mousse (CPP-ACP) on the viability of cultured L929 fibroblasts, which have similar physiological and morphologic characteristics as gingival fibroblasts. The effects on cell proliferation depended on the dilution of Tooth Mousse used. No cell proliferation was observed between 1 and 3 days for dilutions 10^{-3} and 10^{-4} , whereas for the other dilutions tested (10^{-6} , 10^{-8} and 10^{-12}), an increase in cell count was observed within 1–3 days. A so-called ‘toxic threshold’ was observed for longer periods (7 days), the cell count was decreased for dilutions 10^{-3} to 10^{-6} , whereas for the other dilutions (10^{-8} and 10^{-12}), the cell count remained the same as at day 3. Apoptosis was seen for all dilutions except for 10^{-12} . This toxic effect was attributed to the increased amount of calcium and other ingredients and additives present in the Tooth Mousse (glycerol, polypropylene glycol, phosphoric acid, etc.) with higher concentrations of CPP-ACP, interfering with cell proliferation and apoptosis. Furthermore, deposition of CPP-ACP was observed at the cell surface with the highest concentration of Tooth Mousse used, which may explain the toxic effect. This study concluded that low concentrations of CPP-ACP could therefore be used to maintain the viability of the PDL *in vitro*.

7.3. Modification of Shear Bond Stress (SBS)

Another potential application of CPP-ACP is for brackets and modification of the SBS involved. SBS corresponds to the force required to dislodge orthodontic brackets from teeth. Depending on the type of orthodontic brackets, a high or a low SBS is desirable. For ceramic brackets, the SBS should not be too high to avoid damaging the teeth during debonding, whereas for metal brackets, a higher SBS is desirable as the bond must be strong enough in regard to the forces applied during orthodontic treatment [Bishara, 2000]. CPP-ACP has been studied

Fig. 5. Summary of the bioactive properties of CPP-ACP in the oral cavity.

mainly with metal brackets to increase SBS. SBS was evaluated with human [Xiaojun et al., 2009; Baysal and Uysal, 2011; Tabrizi and Cakirer, 2011; Uysal et al., 2011] and bovine teeth [Kecik et al., 2008]. An increase in bond strength was found for bovine teeth when applying CPP-ACP paste, fluoride plus CPP-ACP or acidulated phosphate fluoride as compared to the control teeth where no pretreatment was applied [Kecik et al., 2008]. No significant difference for the SBS was found with the three treatments (CPP-ACP paste, fluoride plus CPP-ACP and acidulated phosphate fluoride). On the contrary, when using human teeth, the fluoride treatment resulted in a decrease in SBS as compared to the control while CPP-ACP and fluoride plus CPP-ACP did not modify SBS. An increase in the SBS was seen in the presence of CPP-ACP when human teeth were previously demineralised fol-

lowing a microabrasion procedure, which may be used in the management of WSL [Baysal and Uysal, 2011]. In another study, pretreatment with CPP-ACP resulted in an increase in SBS for human teeth, or no significant difference as compared to the control was found depending on the type of bonding adhesive used [Xiaojun et al., 2009]. Similar results were found where no significant difference for SBS of bonding systems could be observed between human tooth controls and teeth pretreated with CPP-ACP [Zorba et al., 2010; Uysal et al., 2011]. The pretreatment of the teeth with CPP-ACP is reported to result in a rougher etched enamel surface, which may be responsible for a greater adhesiveness and better bonding of resin tags of the brackets [Xiaojun et al., 2009]. The outcome of these different studies is not clear as they do not agree with each other. This research area investigat-

ing the effect of CPP-ACP on SBS is relatively new and more standardised procedures will help to better understand the impact of CPP-ACP on SBS.

Different bioactive properties of CPP-ACP in the oral cavity have been discussed based on the numerous outcomes of *in vitro* and *in situ* studies. These bioactivities can be classified into four categories including the remineralisation, prevention of demineralisation, anti-plaque and other bioactivities (fig. 5). To date, most studies carried out on the role of CPP in the treatment of dental caries have been conducted on mammalian teeth *in vitro* and *in situ* [Cai et al., 2003; Azarpazhooh and Limeback, 2008; Zero, 2009]. In 1995, it was already reported that most commonly applied methods in dental research had been carried out *in vitro* [White, 1995]. Despite numerous studies carried out *in situ*, a similar situation is found today, as very little has been done *in vivo* so far. The limitation of the *in vitro* models comes from the fact that they do not reproduce the biological intra-oral conditions associated with caries, nor the intra-oral surface area (volume and composition of saliva and tooth surface) and finally, they are associated with artefacts originating from the dental substrate choice (i.e., faster time periods of demineralisation and remineralisation as compared to the *in vivo* situation, dental substrate choice that can affect its reactivity to the protocol applied) [White, 1995]. Some clinical trials demonstrated the anticariogenic role of CPP-CP (calcium phosphate) using rat models [Reynolds et al., 1995; Guggenheim et al., 1999]. Many human intervention studies on the remineralising potential of CPP-ACP have been conducted (table 5). Most of these trials reached the conclusion that CPP-ACP has a positive role in tooth health. Nevertheless, a few studies have reported no benefit of using CPP-ACP [Beerens et al., 2010; Brochner et al., 2011] and other studies obtained results which were contradictory [Altenburger et al., 2010]. The outcomes of these human intervention trials will be discussed in the following section.

8. Contradictory Results on the Remineralising Potential of CPPs

Despite numerous studies dealing with the efficiency of ACP-CPP to combat the development of dental caries and erosion, there is still some controversy on the efficiency of this ingredient. In a recent review addressing non-invasive strategies for tooth repair, Peters [2010] reported the fact that there was a lack of strong clinical evidence on the remineralising effect of CPP-ACP.

8.1. Major Outcomes of Reviews and Meta-Analyses on the Remineralising Potential of CPP-ACP

The publication of several systematic reviews and meta-analyses on the remineralising properties of CPP-ACP demonstrates that the scientific community has a significant interest in the area [Pitts and Wefel, 2009; Reynolds, 2009; Yengopal and Mikenautsch, 2009; Zero, 2009; Cochrane et al., 2010; Gupta and Prakash, 2011]. A systematic literature survey on the tooth remineralising properties of CPP-ACP was conducted [Azarpazhooh and Limeback, 2008]. Out of 10 articles dealing with the remineralising properties of CPP-ACP, 8 were conducted *in situ* and 2 *in vivo*. In 7 of these trials, the same conclusion was reached, showing a remineralisation of the enamel, which followed a dose-response pattern. Nevertheless, 2 studies did not show any additional benefit of utilising CPP-ACP for the prevention of dental caries and 1 study led to conflicting results [Azarpazhooh and Limeback, 2008]. Comprehensive reviews on the remineralising properties of CPP-ACP have suggested a lack of independent scientific research in the area [Azarpazhooh and Limeback, 2008; Yengopal and Mikenautsch, 2009; Zero, 2009]. Most trials concluding in a positive role of CPP-ACP for the treatment of dental caries have been carried out by a research team which has been involved in the patenting of CPP-ACP complexes. Nevertheless, several independent studies leading to a positive effect of CPP-ACP have recently been published as outlined in different review articles [Pitts and Wefel, 2009; Gupta and Prakash, 2011]. However, there are still some conflicting results, making it difficult to draw definitive conclusions regarding the efficacy of CPP-ACP for the prevention of dental caries [Azarpazhooh and Limeback, 2008].

Reynolds [2009] compiled a review to demonstrate the scientific evidence around the utilisation of CPP-ACP in oral hygiene. The scientific evidence was based on a series of facts that have been demonstrated in the literature arising from his research group and from that of other groups. A summary of the evidence reported to date is as follows: (1) CPP-ACP is efficient in remineralising and inhibiting the demineralisation of the tooth enamel subsurface, and (2) CPP-ACP can increase the availability of ions (including calcium) at the surface of the tooth and bind to bacteria localised in the plaque.

In the two reviews from Azarpazhooh and Limeback [2008] and Zero [2009], it was advised to conduct more randomised controlled trials (RCT) in order to determine the long-term effect of the utilisation of CPP-ACP by hu-

man subjects as the first RCT with CPP-ACP had been carried out by Reynolds et al. [2003]. The authors also suggested that more independent studies were needed in the area.

8.2. Evaluation of the Remineralisation Potential of CPP-ACP *in vivo*

A literature search has been conducted for the period 2005–2012 on the databases MEDLINE, Science Direct and Google Scholar. The search terms were as follows: ‘CPP-ACP’, ‘clinical trial’, ‘in vivo’. Additionally, other references were found in different review papers dealing with the remineralising properties of CPP-ACP [Reynolds, 2009; Yengopal and Mikenautsch, 2009; Zero, 2009; Cochrane et al., 2010; Gupta and Prakash, 2011]. This analysis revealed that to date 22 different *in vivo* clinical trials with CPP-ACP have been carried out on human subjects [Reynolds et al., 2003; Kowalczyk et al., 2006; Andersson et al., 2007; Ardu et al., 2007; Milnar, 2007; Gughani et al., 2008; Morgan et al., 2008; Reynolds et al., 2008; Bailey et al., 2009; Caruana et al., 2009; Rao et al., 2009; Zhou et al., 2009; Altenburger et al., 2010; Beerens et al., 2010; Kitasako et al., 2010; Marchisio et al., 2010; Tang and Millar, 2010; Uysal et al., 2010; Baroni and Marchionni, 2011; Brochner et al., 2011; Ferrazzano et al., 2011; Robertson et al., 2011]. Within these trials, 16 (table 5) studied the remineralising properties of CPP-ACP [Reynolds et al., 2003; Andersson et al., 2007; Ardu et al., 2007; Morgan et al., 2008; Reynolds, 2008b; Bailey et al., 2009; Rao et al., 2009; Zhou et al., 2009; Altenburger et al., 2010; Beerens et al., 2010; Kitasako et al., 2010; Uysal et al., 2010; Baroni and Marchionni, 2011; Brochner et al., 2011; Ferrazzano et al., 2011; Robertson et al., 2011].

These clinical trials included follow-up periods of up to several years, i.e. 2 [Kitasako et al., 2010; Morgan et al., 2008] and 3 years [Baroni and Marchionni, 2011]. These studies supported the fact that CPP-ACP have a long-term effect for the prophylaxis of caries and WSL [Yengopal and Mikenautsch, 2009]. Out of the 16 human intervention trials discussed in this section, 5 studied caries regression [Reynolds et al., 2003; Morgan et al., 2008; Reynolds et al., 2008; Rao et al., 2009; Altenburger et al., 2010], while 9 studied the action of CPP-ACP on the remineralisation of WSL appearing after orthodontic appliances [Andersson et al., 2007; Ardu et al., 2007; Bailey et al., 2009; Zhou et al., 2009; Beerens et al., 2010; Uysal et al., 2010; Kitasako et al., 2010; Brochner et al.,

2011; Robertson et al., 2011], 1 studied the effect of CPP-ACP on molar incisor hypomineralisation [Baroni and Marchionni, 2011] and another studied the effect of CPP-ACP on remineralisation of demineralised and non-demineralised human tooth specimens attached to the teeth of human subjects [Ferrazzano et al., 2011].

The localisation of CPP-ACP at plaque level was demonstrated in a RCT where subjects were instructed to chew gums containing CPP-ACP [Reynolds et al., 2003]. In the same study, the positive role of CPP-ACP in the localisation of calcium and phosphate ions in the plaque was shown following the use of a mouthwash formulated with CPP-ACP [Reynolds et al., 2003]. The effect of CPP-ACP on caries regression has been studied in a clinical trial that was conducted on 2,720 teenagers where the efficiency of a sugar-free gum containing 54 mg CPP-ACP was tested versus a sorbitol-based sugar-free gum (control) [Morgan et al., 2008]. During the trial, the subjects brushed their teeth with fluorinated toothpaste and were exposed to fluorinated drinking water. The approximal caries surface reduction was 17% more with the CPP-ACP group as compared to the control group. Additionally, some evidence of remineralisation of both the enamel and dentin was seen through the regression of surface lesions with the CPP-ACP gum. A fluorescent non-destructive technique, DIAGNOdent, was used *in vivo* to monitor early carious lesions [Altenburger et al., 2010]. The application of Tooth Mousse on carious lesions over a period of 3 weeks resulted in a decrease in the fluorescence value compared to a fluoride toothpaste. This decrease was correlated with a reduction of enamel surface porosity [Altenburger et al., 2010]. Nevertheless, no differences could be seen when a visual assessment of the fissures was carried out. Because of these contrasting results, the authors concluded that CPP-ACP may be used as an adjunct for the prophylaxis of occlusal fissures [Altenburger et al., 2010]. Most studies carried out to date have utilised commercial ingredients or products containing CPPs. Very few reported the utilisation of in-house generated CPPs. However, in the RCT conducted by Rao et al. [2009], the CPPs were manufactured at laboratory-scale before being incorporated in a toothpaste. The RCT reported a decrease in decayed surface when CPP-ACP or sodium monofluorophosphate (SMFP) toothpastes were used. Less new lesions occurred in children with the CPP-ACP and SMFP toothpastes as compared to those using the placebo toothpaste [Rao et al., 2009].

Recently, Ferrazzano et al. [2011] developed an *in situ* model using the natural environment of the oral cavity of

human subjects for the evaluation of CPP-ACP efficacy. However, because the subjects wore enamel specimens attached to their teeth using an adhesive procedure, this approach has been classified as an *in vivo* study. Two human enamel specimens which went through a demineralisation procedure were placed on the teeth of healthy adolescents. Topical application of a paste (Tooth Mousse or a placebo) was carried out 3 times daily over a month. The design of this human trial was interesting as it included a control group where the subjects were also equipped with either a sound or a demineralised enamel specimen and were using their normal oral hygiene routine. Additionally, all the conditions tested *in vivo* were also tested *in vitro*. SEM analysis revealed the formation of an amorphous deposit at the demineralised tooth surface after CPP-ACP treatment. The deposit was entirely filling the lesions. This result demonstrated the potential of CPP-ACP as a remineralising agent which could help combat dental caries [Ferrazzano et al., 2011].

The positive role of CPP-ACP as a topical treatment (5-min application) was demonstrated *in vivo* in adolescents with orthodontic brackets [Uysal et al., 2010]. The remineralisation around the brackets was investigated using microhardness analyses with subjects scheduled for a premolar tooth extraction for orthodontic reasons. CPP-ACP allowed remineralisation around orthodontic brackets; the same level of remineralisation was achieved with the application of a fluoride gel [Uysal et al., 2010]. In adolescent subjects, CPP-ACP present in Topacal C5 [Andersson et al., 2007] was applied for 3 months followed by 3-month utilisation of fluorinated toothpaste (1,000–1,100 ppm). This treatment gave better results for the disappearance of WSL as compared to the control group where subjects were instructed to use a fluorinated mouthwash and toothpaste for the same duration. Differences between the test and the control group were already seen after 1-month treatment with a regression of WSL being more marked in the presence of CPP-ACP. The proportion of white lesion sites that totally disappeared was of 55% with the CPP-ACP treatment and 18% for the control after 6 months. These were of 63% (CPP-ACP treatment) and 25% (control) after the 12-month follow-up period. Similarly, another study found that 31% more lesions had regressed with the remineralising cream (Tooth Mousse/MI Paste) than with the placebo at 12 weeks [Bailey et al., 2009]. Reduction in enamel demineralisation was also demonstrated *in vivo* with orthodontic patients after the utilisation of Tooth Mousse [Zhou et al., 2009]. The use of CPP-ACP paste to improve the aesthetic appearance of teeth after bracket debond-

ing was described. A combination of microabrasion and a topical treatment with CPP-ACP paste (Tooth Mousse) resulted in the disappearance of superficial WSL [Ardu et al., 2007]. Similarly, the utilisation of MI Paste Plus after tooth brushing with a fluoride paste reduced enamel decalcification index by 53.5%. This showed the influence of CPP-ACP both by reducing the number of WSL and in their prevention [Robertson et al., 2011]. Recently, Kitasako et al. [2010] developed a micro-pH sensor consisting of a solid state electrode to monitor pH changes directly at the surface of the tooth lesion to study the influence of CPP-ACP treatment in a clinical trial over a 24-month period. Kitasako et al. [2010] monitored pH changes in white spot enamel lesions (WSEL) using a micro-pH electrode in 8 human subjects who received a treatment with MI Paste over a period of 24 months. There was no progression to cavitation of the WSEL and the appearance of the lesions visually improved, possibly due to the remineralising effect of CPP-ACP. The treatment showed an increase in the pH of the WSEL over time (5.94 at 0 month vs. 6.70 after 24 months) toward pH values for sound enamel (6.80 at time 0 vs. 6.83 after 24 months). The pH increase in the WSEL was attributed to either a better access of the saliva to the lesion or to the buffering capacity or remineralisation action of the CPP-ACP in the plaque and the WSEL.

CPP-ACP was shown to improve the appearance of molar incisor hypomineralisation in children who received a daily topical application of GC Tooth Mousse [Baroni and Marchionni, 2011]. The remineralisation of molar incisor hypomineralisation improved over a 3-year period, leading to increased levels of calcium and phosphate. Microscopic analysis also revealed that the enamel structure improved, with a smoother enamel surface and an improved porosity.

In another RCT study no significant effect of CPP-ACP on the improvement of WSL could be demonstrated. Tooth Mousse was used for topical applications on WSL in combination with normal tooth brushing with fluoride toothpaste. The control group brushed their teeth with the fluorinated toothpaste only [Brochner et al., 2011]. The authors found no statistically significant difference in the regression of the lesions between both groups after 4 weeks' treatment (56% mean area decrease in WSL for the group treated with CPP-ACP versus 26% for the control group) despite a trend showing a greater effect of the CPP-ACP treatment. Nevertheless, the relatively short time over which the study was carried out (4 weeks) and the size of the group (60 subjects) may explain why no statistically significant differences were

seen between the CPP-ACP group and the control group. Similarly, no improvement in the WSL size or the plaque composition was seen with adolescents using CPP-ACFP as a topical treatment after debonding of their orthodontic appliances [Beerens et al., 2010].

Within the *in vivo* studies described in the scientific literature (table 5), 5 clinical trials have been conducted by Reynolds' group [Reynolds et al., 2003; Morgan et al., 2008; Reynolds et al., 2008; Bailey et al., 2009; Kitasako et al., 2010] while 11 other trials have been performed by different groups [Andersson et al., 2007; Ardu et al., 2007; Rao et al., 2009; Zhou et al., 2009; Altenburger et al., 2010; Beerens et al., 2010; Uysal et al., 2010; Baroni and Marchionni, 2011; Brochner et al., 2011; Ferrazzano et al., 2011; Robertson et al., 2011]. Around 80% (13 out of 16 *in vivo* human interventions) of the clinical trials reported a positive role of the CPP-ACP complex on the remineralisation of teeth (table 5). The study by Brochner et al. [2011] showed a positive role, but the results obtained with CPP-ACP were not statistically significantly different from the control (fluoride). Similarly, Altenburger et al. [2010] demonstrated a beneficial effect of CPP-ACP on the regression of fissures, but this could only be measured instrumentally and was not confirmed following a visual inspection of the teeth. However, 1 study did not show any additional benefit of CPP-ACFP on the improvement of WSL [Beerens et al., 2010]. In addition, a number of *in vitro* and *in situ* trials also reached the conclusion that CPP-ACP fails to remineralise tooth surfaces [Schirrmeyer et al., 2007; Rehder Neto et al., 2009; Lata et al., 2010; Wegehaupt and Attin, 2010; Wang et al., 2011; Wegehaupt et al., 2011].

8.3. Possible Origin of the Contradictory Results

Contradictory results seem to arise mainly from the methodology utilised to conduct trials, notably the type of dental appliances used. According to Zero [2009], the studies conducted by Reynolds' group were carried out in conditions that are not representative of demineralisation/remineralisation situations in the human mouth. The enamel slabs on the appliances were not positioned in caries-prone tooth sites as these were in contact with the tongue (palatal appliances). The location of the appliances affects the degree of remineralisation, as contact with chewing gum varies depending on the location of the teeth. This may be the reason why the results observed in an *in situ* experiment where the appliances were positioned in mandibular appliances were different. This

study showed no benefit of chewing gums containing CPP-ACP on tooth remineralisation as there was no direct contact of the chewing gum with the lesions [Schirrmeyer et al., 2007]. Furthermore the term CPP-ACP has been incorrectly utilised in a number of instances. For example, Schirrmeyer et al. [2007] used chewing gums containing casein/hydrolysed casein and calcium phosphate instead of CPP-ACP [Reynolds, 2009]. Other differences may arise from the depth of the lesions used. These were twice as deep in the study by Shen et al. [2001] as compared to the study by Schirrmeyer et al. [2007]. Deeper lesions may allow the better detection of differences in terms of remineralisation. Another point is the relatively large standard deviation associated with the remineralisation measurements in the Schirrmeyer et al. [2007] study, making it difficult to interpret the results.

The manner in which appliances are utilised affects the remineralisation results. When appliances were removed during food and drink intake, this excluded the possibility to study the effect of acid erosion [Zero, 2009]. Nevertheless in a recent *in situ* study, enamel lesions presenting a plaque biofilm were subjected to frequent acid challenge induced during the consumption of sugar confections and remineralisation was still demonstrated in these conditions when CPP-ACP was added to the confectionaries even at levels as low as 0.5% (w/w) [Walker et al., 2010].

Variation may also arise from the treatment applied to the control group during different remineralisation trials. In 2 *in vivo* human intervention studies, the control groups used deionised water [Reynolds et al., 2003, 2008], which may have caused a demineralisation of the teeth and thus affected the outcome of the studies. Zero [2009] raised the concern that most of the studies from Reynolds' group have been carried out with CPP-ACP and fluoride. Nevertheless, Reynolds [2009] reported in his review that Recaldent has been developed to be used in combination with fluoride as an adjunct for treatment of early-stage caries development. This follows the suggestion of Pfarrer and Karlinsey [2009], who proposed that calcium-containing technologies developed for tooth remineralisation must be used to augment fluoride action in the treatment of tooth decay and that possible interactions with fluoride should be addressed in order to maintain its activity. This wide variability in the protocols developed so far suggests that there is a need for more standardisation in the methodologies employed to assess CPP-ACP remineralising properties [Pitts and Wefel, 2009].

In their meta-analysis, Yengopal and Mickenautsch [2009] reported that in all the studies where lesions have been exposed to CPP-ACP, a significant improvement of remineralisation in respect to control lesions was observed. Similar conclusions were drawn by Gupta and Prakash [2011] in their recent comprehensive review on CPP-ACP. Results obtained *in situ* suggest a short-term effect of the remineralisation observed with CPP-ACP [Yengopal and Mickenautsch, 2009]. The effective dose for short-term trials has been found to be between 10 and 18.8 mg of CPP-ACP in sugar-free gums, whereas long-term studies have utilised a higher dose of CPP-ACP, i.e., 54 mg in sugar-free gums [Yengopal and Mickenautsch, 2009].

In summary, clinical trial outcomes seem to suggest the prophylactic role of CPP-ACP in the improvement of oral health in agreement with the numerous studies carried out *in vitro* and *in situ* [Gupta and Prakash, 2011]. However, it appears that stronger evidence is still needed to unequivocally recommend CPP-ACP in the non-invasive treatment of dental caries [Peters, 2010; Gupta and Prakash, 2011]. Nevertheless, CPP-ACP has significant potential as an adjunct to fluoride in the prevention of caries [Peters, 2010; Gupta and Prakash, 2011]. The conflicting scientific evidence in relation to the tooth remineralising properties of CPP-ACP may be related to differences in experimental protocols and in the relatively large variability found in some studies, thus making interpretation of the results obtained challenging. An interesting *in vivo* model has recently been developed by Ferrazzano et al. [2011], where human teeth have been fixed onto triallist teeth in order to study the remineralisation potential of CPP-ACP. The advantage of this model is that all attached teeth had previously gone through the same demineralisation procedure while in constant contact with the human oral cavity during the time frame of the study. This new model may help to provide more reproducible data on the effect of CPP-ACP *in vivo*. At present, very little is known about the long-term effect of CPP-ACP in humans. The longest clinical trial reported to date is 3 years and the longest follow-up period is 24 months. There is a need to generate more knowledge on the possible long-term consequences of such ingredients on human oral/dental health.

9. Conclusions

The role of CPP's has been discussed using a wide range of *in vitro*, *in situ* and *in vivo* studies to assess their bio-functionality mainly as mineral carriers in the oral cavity.

CPPs play a role in the remineralisation process of tooth enamel, allowing the repair of lesions that can occur at the enamel and dentin level. The enamel formed in the presence of CPP-ACP appears to be more resistant than normal enamel. This observation is very interesting from the perspective of further slowing down or preventing the demineralisation process. A protective effect of CPP-ACP has been seen when incorporated into foods with erosive properties such as high sugar confectionary and low pH soft drinks. They have been successfully incorporated in a wide range of carriers ranging from oral hygiene products (toothpaste, mouth rinse), adjuncts for oral hygiene (chewing gums and lozenges) to actual foods.

The application of CPPs as dental health enhancement agents presents a major advantage over other bioactive peptides. Since their site of action is located in the mouth, it is less likely that they will be degraded as they can act as soon as they enter the oral cavity. A number of studies have nevertheless found contradictory results, showing no bioactivity of CPPs in the oral cavity or no synergistic action between fluoride and CPP-ACP. These differences may arise from the methodology employed to assess CPP bioactivity. The protocols employed should represent the conditions occurring in the oral cavity to extrapolate the efficiency of CPPs when they are used by human subjects. The outcomes of different studies suggest that the residence time and an effective contact of CPP-ACP with the teeth may be crucial parameters in order to observe beneficial effects with CPPs. Therefore, the carrier employed for delivery of CPP-ACP can be an important factor in their bioactivity. A large amount of data has been generated on the efficacy of CPP-ACP in different carriers including foods. Nevertheless, very little is known about interactions between CPP-ACP and other food components in the oral cavity. In other words, the effect of a given diet or specific food consumption on CPP-ACP bioactivity is still not very well understood. This aspect needs to be addressed to try to better understand the source of variation seen in different intervention trials conducted in humans.

Various human intervention trials have been carried out evaluating the role of CPP-ACP on the remineralisation of enamel. Most of these trials (13 out of 16, which accounts for 80%) reached the conclusion that CPP-ACP has a positive role in tooth remineralisation. The generation of universally accepted guidelines for the design of future human intervention trials may allow better comparison of different studies. It was earlier outlined, for example, that the residence time of CPP-ACP may significantly affect their efficacy. However, very little infor-

mation in this regard has been provided in many of the studies performed to date, while this parameter may be the key for bioactivity in the oral cavity; additionally, there is a need to generate more knowledge on the possible long-term consequences of such ingredients on human dental health. Although one could argue that CPPs are naturally formed within the human body, they generally appear in the gastrointestinal tract following milk consumption. Their addition in food and oral hygiene products consequently increases their concentration to a level not normally observed in the oral cavity of humans. CPPs' effect on other tissues in the mouth may need to be studied in more detail. Initial data suggesting that they can display cytotoxicity at high concentrations on periodontal ligament cells and affect the viability of fibroblasts merit further study. Therefore, specific regulations may need to be developed regarding acceptable dosages of these ingredients in foods and oral hygiene products.

The health issue represented by caries development and erosion seems to be correlated in most cases with poor oral hygiene practices. This issue needs to be tackled at source. A more focused development of public policies involving the producers of oral hygiene products and adjuncts for oral hygiene could be beneficial in order to stress the importance of good oral hygiene procedures

from an early age to ensure the development of lifelong healthy habits in the population. CPP-ACP can be used in parallel to help prevent the development of dental caries and erosion. This may be potentially relevant for those segments of the population, such as toddlers, infants and elderly, where brushing of teeth can be a challenging task. In addition, the use of CPP-ACP in combination with fluoride may have potential to help avoid fluorosis in infants while combatting the development of dental caries.

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