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Review



Assessing *in vitro* remineralization of primary artificial caries: A systematic review of multi-techniques characterization approaches

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ABSTRACT

Objectives: The aim of this systematic review is to help the reader in their selection of analytical techniques for a complete and accurate assessment of artificial caries remineralization.

Data, sources and study selection: Four databases were scanned independently following PRISMA guidelines and, after setting of inclusion and exclusion criteria, 255 potentially-relevant articles were scrutinized for selection. Based on our hypothesis that at least three characteristics of the newly-formed mineral, i.e. spatial extension, chemical composition and morphology, are requested for assessment, focus was made on 24 articles where at least three methods were used.

Conclusion: Twenty different analytical methods were used and 11 studies used at least one technique relevant to each of the three characteristics. However, there was a general lack of quantitative and chemical characterization of the new mineral. These results highlight the scarcity of multi-techniques studies and the low diversity of techniques currently used in this area. We suggest that the combination of qualitative micro computed tomography combined with X-Ray diffraction or vibrational spectroscopies is the most suitable to fully assess the success of artificial caries remineralization.

Clinical significance: Modern prospective caries studies require the measurement of small changes in tooth mineral content. This can probably be achieved only by combining several diagnostic tools. Artificial caries allows for a primary practical assessment of such combinations. However, it is important to improve the awareness and/or availability of some relevant analytical tools and to better connect laboratory and clinical objectives.

1. Introduction

During the past few decades, scientific developments in cariology, dental materials, and diagnostic systems have changed dentistry's approach for the diagnosis and management of dental caries. Minimally-invasive dentistry is based on maintenance of health, early detection and surveillance, control of causal agents, and use of appropriate pharmacological agents with minimal intervention, with the objective of preserving the dental tissue [1].

The concepts of minimal intervention dentistry are supported by a better understanding of caries development mechanisms. However, the large variability in activity status, size, as well as structural differences within different carious zones make such investigations difficult. In this

respect, *in vitro* models are the most commonly employed methods in cariology research [2]. This type of models has been helpful to understand the etiology and pathogenesis of this disease, evaluate preventive agents and develop methods of caries elimination. Various methods have been used to produce artificial caries, mainly via chemical [3] or biological methods [4]. Among these protocols, pH-cycling models are particularly interesting as they involve exposure of dental substrates to successive steps of demineralization and remineralization, thus closely mimicking caries evolution in mouth conditions [5].

There is often confusion in the literature in the terminology used for caries detection and caries diagnosis [6]. In the last decade, three terms have been agreed on: lesion detection, implying an objective method of determining whether or not disease is present; lesion assessment, aiming

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to characterize or monitor a detected lesion; and caries diagnosis, that should involve a human, professional, summation of all available data [7]. For the assessment of initial caries, the main criterion is the measure of the lesion depth. The method used for such measurement is therefore a critical factor, all the more as there is a clinical requirement to detect carious lesions at their earlier stage. Moreover, as part of the minimal intervention dentistry, some authors recommend excavating only the outer layer of highly infected, denatured dentin and preserving its intact, bacteria-free, remineralizable inner layer [8]. Thus, the measurement method has to be sensitive enough to distinguish these different layers.

However, assessing remineralization strategies success should require a more complete characterization of the newly-formed mineral. Beyond the spatial information, it is important to check the similarity of the new phase with that of the native tissue in terms of composition and structure. Numerous methods have been reported to characterize the optical, mechanical, structural and chemical properties of demineralized and remineralized enamel and dentin [9]. Indeed, a single technique is unlikely to provide all needed information. However, the large number of available studies involving a wide variety of analytical methods makes it difficult to decipher what are the most suited methods for such studies.

The aim of the current systematic review is to help the reader in their selection of techniques for the assessment of artificial caries remineralization, by answering the focus question: "Which are the most suited analytical methods to assess successful demineralization and/or remineralization of artificial carious lesion created in enamel or dentin?". Based on our hypothesis that at least three characteristics of the newly-formed mineral, *i.e.* spatial extension, chemical composition and structure, are requested for such an assessment, focus has been made on the available literature where at least three methods were used.

2. Methods

2.1. Protocol and registration

This systematic review conformed to Preferred Reporting Items for Systematic Reviews and Meta-Analyses PRISMA Checklist [10]. The protocol was registered in the international Prospective Register of Systematic Reviews (PROSPERO) under number CRD42023393813.

2.2. Eligibility criteria

We included experimental studies that evaluate artificial dental caries. Overall, the inclusion criteria were based on the PECOS methodology [11]: Population (P): Teeth; Exposure (E) Artificial dental caries; Comparison (C): No comparison; Outcome (O): Techniques to evaluate artificial dental caries (S): *In vitro* and *in vivo* studies. No data, sex or language restrictions were applied to the search strategy. The exclusion criteria encompassed: (1) Studies with no information on their protocols; (2) *In vivo* studies, literature reviews, intervention studies, letters, case reports and personal opinions; (3) Studies which used secondary caries as dental model, and (4) Studies that used only a single or two techniques for caries detection.

2.3. Information sources and search strategy

Detailed individual search strategies were developed, in English, for each bibliographic electronic database: PubMed (including Medline), Embase, Scopus and Web of science. All database searches were conducted from the starting coverage date through June 31st 2022. More information on the search strategies is provided in **Appendix 1** (Supplementary Material). All references were managed and the duplicated hits were removed by a reference manager software (EndNote X7® Basic-Thomson Reuters, New York, USA).

2.4. Selection process and data collection process

This part followed a two-phase process. In phase one, two authors (T. C. and J.B.) independently evaluated the titles and abstracts of all identified electronic database citations. In phase-two, the same authors evaluated full-text data. They independently screened papers on phase one and two, applied the eligibility criteria, collected key information from the selected studies, and crosschecked the information. The references of these papers were also checked to include any interesting papers that were missed during the search. The final selection was based solely on full-text assessment of the studies. When disagreement appeared, a third author (A.L.P.) was involved to make a final decision about the inclusion or exclusion of studies. The selection phase were managed by Rayyan on line software (www.rayyan.ai)

2.5. Data items

Pre-specified data elements were identified from each study which included the technique of demineralization, the study design, sample size, time, demineralization solution, characterization techniques, time. Data was collected independently by both investigators and entered into Table1.

2.6. Study risk of bias assessment

The methodology of the selected studies was assessed using the Critical appraisal tool for *in vitro* studies, adapted from CRIS Guidelines. Two reviewers (J.B and T.C) had independently scored each item as "yes", "no" or "unclear", and classified independently the quality of each included study as "high", "low" or "moderate" risk of bias. The same two reviewers worked out on any differences regarding data analysis. A third author (A.L.P) was involved to steer decision in case of uncertainty. Figures of the quality assessment of all included studies were generated with Review Manager 5.3 (RevMan 5.3, The Nordic Cochrane centre, Copenhagen, Denmark).

2.7. Risk of bias across studies and reporting bias assessment

The risk of bias across studies was assessed as an overall risk the study results may present, on which could influence meta-analysis data. Methodological and statistical heterogeneity were evaluated by comparing the variability in study design and the risk of bias. When the required data were not complete, the reviewer (J.B) attempted to contact the study authors to retrieve any unpublished information. Three attempts were made in a 30 days' period, by email for the first, second and last author.

2.8. Effect measures

We considered the results in terms of structural, optical, mechanical, and chemical properties of demineralized enamel and dentin as quantifiable markers of caries depth.

2.9. Synthesis methods

If quantitative synthesis was deemed appropriate, a meta-analysis would have been performed by using RevMan 5.3. However, there were not enough data to perform a meta-analysis.

2.10. Certainty assessment

A summary of the overall strength of evidence available was presented using "Grading of Recommendations Assessment, Development and Evaluation" (GRADE) Summary of Findings (SoF) tables, using GRADEpro software.

3. Results

3.1. Study selection

Our initial database searches identified 588 studies. After eliminating duplicated hits, 459 studies remained of which 234 were excluded after title and abstract review, resulting in 225 articles. No additional study was selected following hand-searching of the reference lists of the included studies. During phase-2, 201 studies were excluded, leaving 24 studies for qualitative and quantitative synthesis. A flowchart of the process of identification, inclusion and exclusion of studies, and reasons for exclusion is shown in Fig. 1.

3.2. Risk of bias assessment

According to the appropriate tool, 11 studies were classified as high, 12 as moderate, and another one as low risk of bias (Appendix 2, Supplementary Material). The main methodological bias was related to the calculation of sample sizes and, in some studies, the *in vitro* study did not simulate the *in vivo* situation.

3.3. Results of syntheses

A meta-analysis was not performed because of the insufficiency of data for statistical pooling related to the sort of study included.

3.4. Certainty of evidence

The overall quality of evidence identified using GRADE's SoF tables was assessed as very low (Appendix 3, Supplementary Material), because in some studies the *in vitro* study does not simulate the *in vivo* situation, allowing a high risk of bias. Furthermore, study design labeled as observational and *in-vitro* studies consistently downgraded the certainty.

3.5. Study characteristics and results of individual studies

In the total 225 reports, 45 different methods are reported (Appendix 4, Supplementary Material). Only 5 of them have an occurrence

> 10 %: microhardness (incl. Vickers and Knoop hardness for surface and transversal analyses, 31 %), Scanning Electron Microscopy (SEM, 22 %), Transverse MicroRadiography (TMR, 17 %), (Polarized) Light Microscopy ((P)LM, 16 %) and Energy Dispersive X-Ray spectroscopy (EDX, 13 %) while 25 show an occurrence < 1 % (*i.e.* cited once or twice). Of notice, several methods are used for ion titration in solution, especially electrochemical measurement of fluoride, that in total account for 17 % of occurrence.

Among these, 97 reports use only one method and 104 reports include two methods. In this last category, the most frequent pair of techniques is SEM/EDX (18 %) (Appendix 5, Supplementary Material). Associations of microhardness with SEM, TMR, PLM or ion titration account for 26% of reports.

Finally, 24 studies include at least 3 methods: 15 used 3 methods, 6 used 4 methods, 2 used 5 methods and 1 used 6 methods. All these studies except one were devoted to remineralization strategies. Microhardness is used in 50 % of the studies and the most common triad is microhardness/SEM/EDX (20 %). These studies are gathered in Table 1 and are the focus of this systematic review.

3.6. Artificial caries formation and remineralization procedures

3.6.1. Specimens

Most studies focused on enamel tissue. Samples were prepared as mm size blocks that were ground and/or polished [12–23], except for four studies where full or half-crowns were used [24–26]. When dentine was studied, blocks were mainly prepared from root [27–29], but two studies used crown dentine [30,31]. Only one study used sections including both tissues [32]. In all cases, specimens were partly embedded in a resin or coated with an acid-resistant varnish to restrict the localization of caries as well as to obtain control tissue for comparison.

3.6.2. Artificial caries formation

All studies but five used a chemical method to induce caries. Typical demineralizing solution contained calcium and phosphate ions in the mM concentration range in a 0.05 or 0.075 M acetate buffer. The final pH, that was sometimes adjusted with KOH [33], ranged from 4.3 to 5. In some cases, this solution was supplemented with F^- [23]. In one

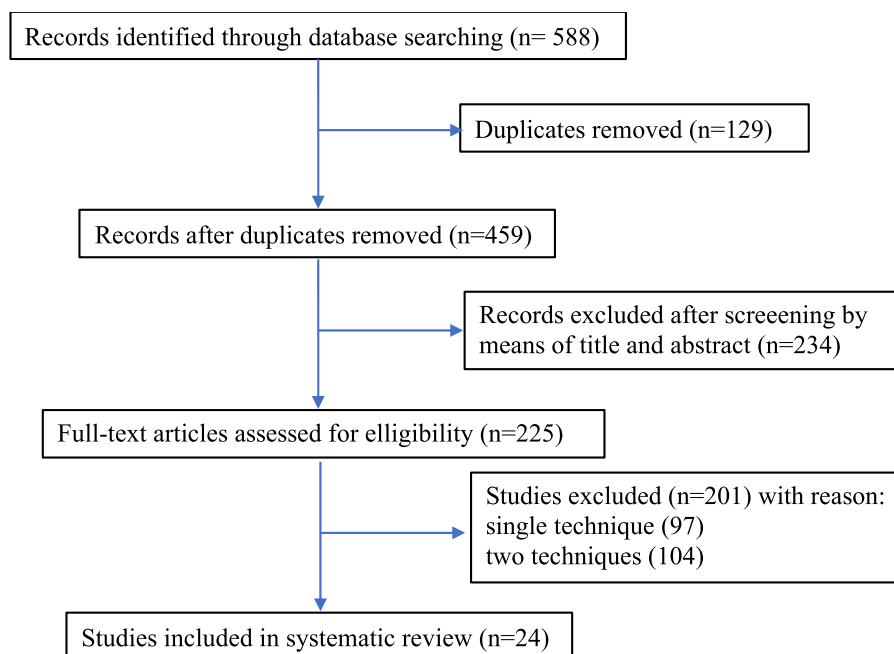


Fig. 1. Flow diagram of literature search and selection criteria.

Table 1

Overview of the 24 articles selected for systematic study: Studied tissue, Demineralization and Remineralization method, Analytical methods for mineralization extent, chemical, surface morphology and miscellaneous characterization.

Refs.	Tissue	Demin.	Remineralization	Mineralization extent	Chemical	Surface	Miscellaneous
Arthur 2015	Dentine	Biol	Toothpastes and mouthwash	KHN, TMR	Raman		CFU
Bakry 2014	Enamel	Chem	Bioglass		EDX, XRD	SEM	
Bilje 2018	Enamel	Chem	Solution	microCT	ICP-OES, EDX, FTIR		
Epasinghe 2015	Dentine	Chem	Toothpastes	KHN, CLSM, TMR			
Epasinghe 2016	Dentine	Chem	Flavonoids solutions	KHN, CLSM, TMR			
Epasinghe 2017	Dentine	Chem	Toothpastes + flavonoid	CLSM, TMR	XRD		Colorimetry
Ferrazzano 2007	Enamel	Chem	Casein phosphopeptide	Colorimetry, Weight		SEM	
Ferrazzano 2008	Enamel	Chem	Casein phosphopeptide	Colorimetry, Weight		SEM	
Goncalves 2021	Enamel	Chem	Toothpaste + trimetaphosphate	KHN, PLM	EDX	CLSM, SEM	
Ibrahim 2020	Enamel	Chem	Sealants	KHN, PLM	EDX	SEM	
Jones 2006	Enamel	Chem	Fluoride solution	PLM, PS-OCT, TMR			
Liu 2019	Dentine	Biol	Silver/fluoride solutions	microCT		SEM	CFU, CLSM
Melo 2013	Enamel	Biol	Fluoride composites/cements	KHN	EDX, Electrochem		
Niu 2021	Dentine	Biol	Antimicrobial peptide	microCT	FTIR, XRD	SEM	CFU, CLSM
Oliveira 2019	Enamel	Chem	Fluoride gels, foam and varnish	KHN	EDX	CLSM, SEM	
Peric 2014	Enamel	Chem	Toothpastes	VHN	EDX	SEM	
Sadoon 2020	Dentine	Chem	Cements	PLM, VHN	EDX		
Savas 2016	Enamel	Chem	Fluoride solutions	CSLM, VHN	EDX		
Sreekumar 2019	Enamel	Chem	Toothpastes	VHN	EDX	SEM	
Taneja 2019	Enamel	Chem	Toothpastes + theobromine		EDX	SEM	Diagnodent
Tulumacci 2019	Enamel	Chem	Toothpastes	PLM	XRD		Diagnodent
Wang 2016	Enamel	Chem	Toothpastes	KHN		AFM, SEM	
Xiao 2015	Enamel	Chem		FIRE, PLM			QLF
Yin 2020	Dentine	Biol	Fluoride /silver nanoparticles	microCT	FTIR, XRD	SEM	

example, lactic acid was used instead of acetic acid and the pH was 3 [25]. Immersion times varied from 6 h to 9 days. Main alternative procedures involve mixture of lactic acid and KOH or NaOH with a polymer (Hydroxyethylcellulose, Carboxymethylcellulose, Carbopol) to obtain an acidic gel [17,34]. Finally, in one study, the McInnes solution, consisting of HCl, H₂O₂ and ether, was impregnated on a cotton and applied 2 times 3 min at 24 h interval [20].

Caries induction by a biological method involved the incubation of the specimen with *Streptococcus mutans* alone [31], *S. mutans* and *Lactobacillus casei* [35] or with a mixture of 6 cariogenic bacteria strain [29]. Growth media were all supplemented with sucrose and, in one case, with saliva. Incubation times varied from 24 h [29] to 5 days [13].

3.6.3. Remineralization procedures

Remineralization of chemically-induced artificial caries are usually performed using a pH cycling approach (Fig. 2). For this a remineralization solution, consisting of calcium and phosphate ions in the mM concentration range, KCl ca. 0.1 M and, in most cases, an organic buffer (Tris, HEPES, cacodylate) is prepared, with a final pH of 7. In some cases, fluoride ions are also present [12,29]. In one study, collagenase is

added to mimic the degradation of the organic matrix [27]. Cycling consists in the successive contact of the sample with either the demineralization or the remineralization solution. Between 1 and 6 cycles are performed per day, the demineralization step being often the shortest, and the overall experiment can last from 6 days to 4 weeks. Main variations include the use of human or artificial saliva as the remineralization solution or performing a single step of remineralization for up to 30 days. When evaluating the remineralizing efficiency of specific formulations, these are commonly applied between the demineralization and remineralization steps, either once or repeatedly. A similar pH cycling approach has been used for biologically-induced caries. Alternatively, after demineralization, the considered treatment was applied and the sample was studied as such or after being left in a simulated oral fluid.

3.7. Techniques

As previously mentioned, assessing remineralization strategies of artificial caries requires a *minima* to establish the extent of remineralization and to assess the nature of the newly-formed mineral. These analyses should preferentially be performed over the whole volume or at

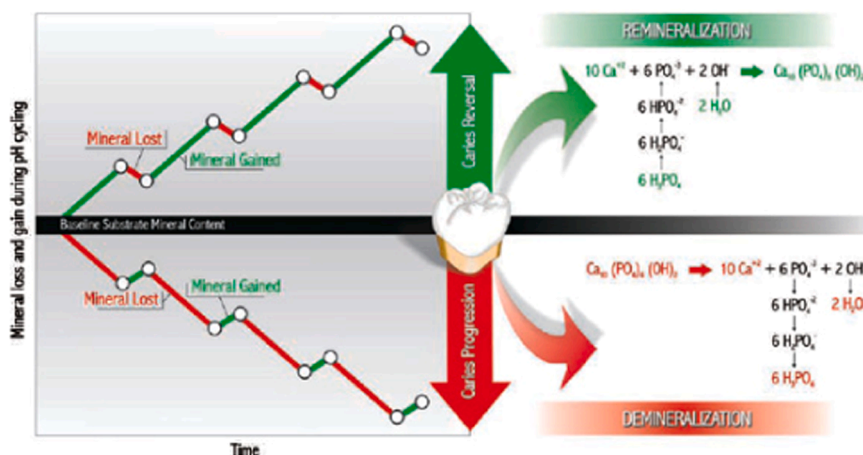


Fig. 2. The pH-cycling process for demineralization-remineralization studies. Reproduced from [36] – CC BY 4.0, based on the original figure of White et al. [37].

least depth of the carious area but, in some cases, surface analyses are used as surrogate or complimentary methods. Miscellaneous, often study-specific techniques can provide additional information.

3.7.1. Extent of remineralization

Extent of mineralization should include quantitative (*i.e.* how much) and spatial (*i.e.* where) information. Spatial information can be gained from imaging techniques. 3D imaging is usually performed using μ -CT (Micro-Computed Tomography) which gives access to the volume of remineralization [32]. Micro-CT records X-Ray transmission through the sample at different angles using a micro-focused source. 2D projections are recovered that can be combined to obtain a 3D view of the sample. Since X-Ray transmission depends on both the atomic weight of the constituting elements and the density of the material, it easily discriminates a mineral phase from soft tissues and can also evidence the microstructure of the sample. By extrapolation, it is also possible to calculate the modulus of elasticity of examined structures [38]. Thus, Micro-CT offers the opportunity for a qualitative and quantitative evaluation of dental hard tissues [39]. The determination of mineral densities by quantitative assessment is of particular use when studying incipient carious lesions and remineralized areas [40] (Fig. 3).

3D images are also obtained using PS-OCT (Polarization Sensitive Optical Coherence Tomography) that records the reflectivity of an infrared light by the sample [12]. In simple OCT, the reflected light is analyzed using an interferometer that allows to obtain images for different depths of the sample. In PS-OCT, polarizations of the incoming and reflected lights are also compared, enhancing the contrast between the mineral phase, which is birefringent, and organic tissues [41].

Confocal Scanning Laser Microscopy (CSLM) may also be used for 3D imaging. CSLM is a fluorescence microscopy technique that records light emission under a focused scanning laser beam at a fixed focal plan, *i.e.* at a selected depth of the sample. This yields to 2D images that can be stacked to obtain 3D images. In the case of dental tissue however, the imaging depth is limited so that CSLM is most often performed after staining a tissue section with a dye that binds to the organic component of the tissue, allowing for imaging the demineralized area [33] (Fig. 4).

Alternatively, 2D imaging using TMR, SEM or (P)LM allow for

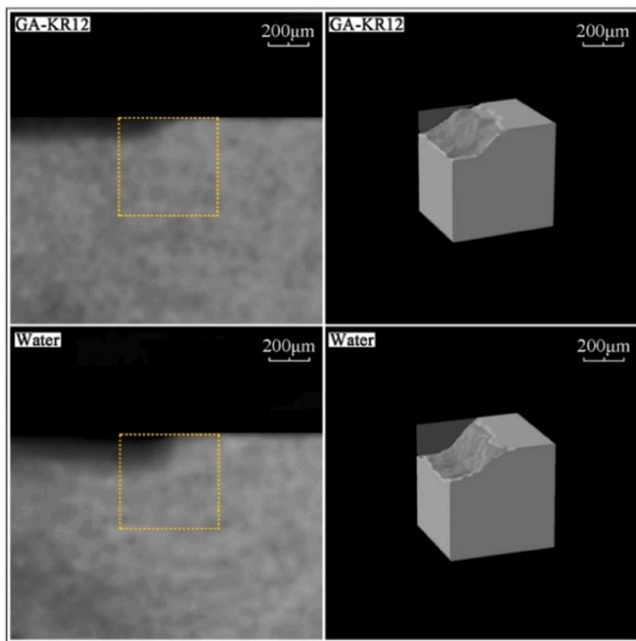


Fig. 3. Micro-CT (left panel) 2D and (B) 3D (right panel) images of biologically-induced artificial lesions remineralization in the presence of the GA-KR12 peptide or not (Water). Reproduced from [31], Copyright (2021) with permission from Elsevier.

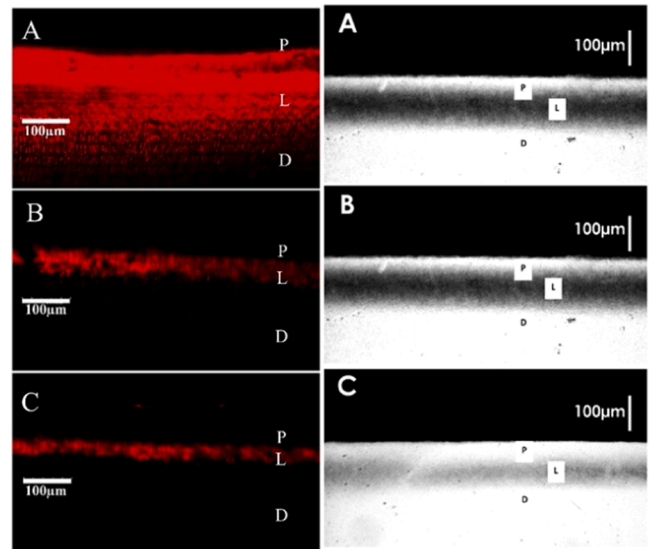


Fig. 4. Comparison of CSLM (left panel) and TMR (right panel) images of artificial lesions after pH cycling (A-control, B-in the presence of Tricalcium Phosphate and Fluoride, C- in the presence of Tricalcium Phosphate, Fluoride and Proanthocyanidin). P: precipitate; L: lesion; D: dentine. Reproduced from [33], Copyright (2017) with permission from Elsevier.

measurement of the lesion depth on transverse sections. Currently, TMR is considered as the gold standard two-dimensional (2D) technique for the determination of Mineral Density *in vitro*. TMR assesses the presence of the mineral via X-Ray light transmission through a section ca. 100 μ m in thickness. The mineral concentration profile is used to calculate lesion depth and integrated mineral loss or gain after remineralization [33] (Fig. 4). Interestingly, the development of wavelength-independent microradiography, which used a polychromatic X-ray beam, has greatly improved the sensitivity of the technique and allow to study much thicker sections [42].

SEM allows for imaging the sample by recording back-scattered by and/or emitted electrons from its surface when irradiated with a scanning electron beam [19,26]. Back-scattered electron (BSE) imaging can be used to study the calcification state of tissues because image contrast is a function of the variations in the average atomic number, which is higher for mineral components than for organic components and water. The gray-level intensity in the BSE image has been reported to be highly correlated with calcium content, mineral content and mineral density [43]. LM relies on light transmission through the sample and the use of polarized light enhances its specificity towards mineral phases. PLM allows the visualization of areas with different porosities and birefringence. In water or conventional mounting media, dentin birefringence results from the strong negative birefringence of the mineral network and the weaker positive birefringence of collagen [44]. As demineralization occurs, birefringence increases by reduction of the mineral contribution. Thus, PLM can give high degree of differentiation between normal, demineralized and remineralized area of tooth sample [21].

Indirect determination of lesion depth can be achieved using transverse (or cross-sectional) microhardness [35]. In carious dentine, a decrease in the mechanical properties is assumed to be associated with a reduction in its mineral content. Assessment of mechanical properties of remineralized dentin is particularly useful, as it is hypothesized that recovery of mechanical properties is achieved when minerals are effectively re-associated with the organic matrix but limited when new minerals are poorly attached to the matrix [45]. Measurements are performed at increasing distances from the surface and the boundary of the lesion is set when the hardness value is comparable to that of sound tissue [28]. Hardness characterizes the resistance of a material to a localized plastic (*i.e.* irreversible) deformation. Most commonly,

hardness refers to indentation hardness where deformation is performed by compression using a diamond-made sharp object. The imprint of the probe is measured and a hardness value is calculated. Microhardness corresponds to hardness measured at low load (< 10 N). Knoop (KHN) and Vickers (VHN) microhardness testing are the two most common methods, that differ in the shape of the probe [46]. Such a difference in shape has an impact on the indentation phenomenon so that Knoop testing is usually recommended for brittle (*i.e.* easily cracking) and/or thin materials and was the most frequent here.

Some specific fluorescent techniques can also be included in this first section. QLF (Quantitative light-induced fluorescence) and FIRE (Fluorescence Imaging with Reflectance Enhancement) both image autofluorescence of caries and compare them to that of sound tissue. QLF detects and quantifies the loss of fluorescence associated with demineralization [47]. The technique is based on the excitation of the dentine with blue light (370 nm) that makes it fluoresce into the yellow-green region. When a lesion is present, an increase of light scattering decreases recovered fluorescence intensity, making the lesion appearing as a dark spot on a bright green background. The loss of fluorescence is obtained by comparison with the fluorescence of healthy enamel (Fig. 5). The main difference between QLF and FIRE is that, in the latter case, white light reflectance at the sample surface is also measured and combined with fluorescence data, enhancing the contrast between sound and damaged tissue [15].

Most of these techniques allows for a qualitative assessment of remineralization extent by comparing the measured property with that of sound tissue. A more quantitative value can be obtained from micro-CT data using a reference mineral sample. The calcium content of the remineralization solution after treatment, as determined by colorimetric titration, was also used as an indicator of mineral precipitation [24,34]. However, the only direct quantification method described in the selected papers relies on the use of a sensitive scale to weight the samples before and after remineralization [24,34].

3.7.2. Chemical characterization

The mineral phase of enamel and dentine is a calcium phosphate

phase, derived from hydroxyapatite. Only X-ray diffraction experiments can unambiguously identify the presence of this phase within the newly-formed mineral [22]. When a X-Rays beam meets an atom, it can propagate in multiple directions. If several atoms are present, some of these diffracted beams can combine in an additive manner (interference). If these atoms are organized in a periodic manner, this combination will be maximum in one direction that is characteristic of this periodicity. The chemical nature of the atom also influences the intensity of the resulting peak. X-Ray Diffraction (XRD) experiments record the intensity at various angles and the whole set of detected peaks is characteristic of one chemical compound in a given crystalline state. In addition, because diffraction reflects the order of the atoms, the width of the diffraction peaks can provide an indication of the crystallinity of the mineral [49].

Vibrational spectroscopies, *i.e.* FTIR (Fourier Transform InfraRed) and Raman, are also reliable techniques to identify the deposited mineral [22,35]. Similarly to UV-visible spectroscopy, FTIR is based on the absorption of light of varying energy. However, infra-red light energy range corresponds to the energy allowing the vibration of two or few atoms bound together. Indeed, this energy depends on the nature of the atoms and their bond length/strength. Therefore, a FTIR spectrum show chemical bonds that exist in a given sample, allowing to identify its chemical nature. Raman spectroscopy also allows for chemical identification via molecular vibrational signatures but by analyzing scattered light. Most relevant vibration peaks are related to those of the phosphate and carbonates groups as well as the amide I bond because their ratio provides an indication of the mineral composition and mineral/organic matrix relative ratio, and therefore of the mineralization extent [50] (Fig. 6.). However, because the two spectroscopies are based on different optical phenomena, the shape and intensity of a peak for a given vibration can vary from one technique to the other. For instance, for human bone, the most intense peak of both FTIR and Raman spectra corresponds to a vibration of the phosphate group. However, in Raman, this peak is narrow, making it a good probe of the hydroxyapatite phase, whereas the amide vibration peaks are at the best 10 times less intense, hindering a precise analysis of the proteins. In contrast, the main FTIR

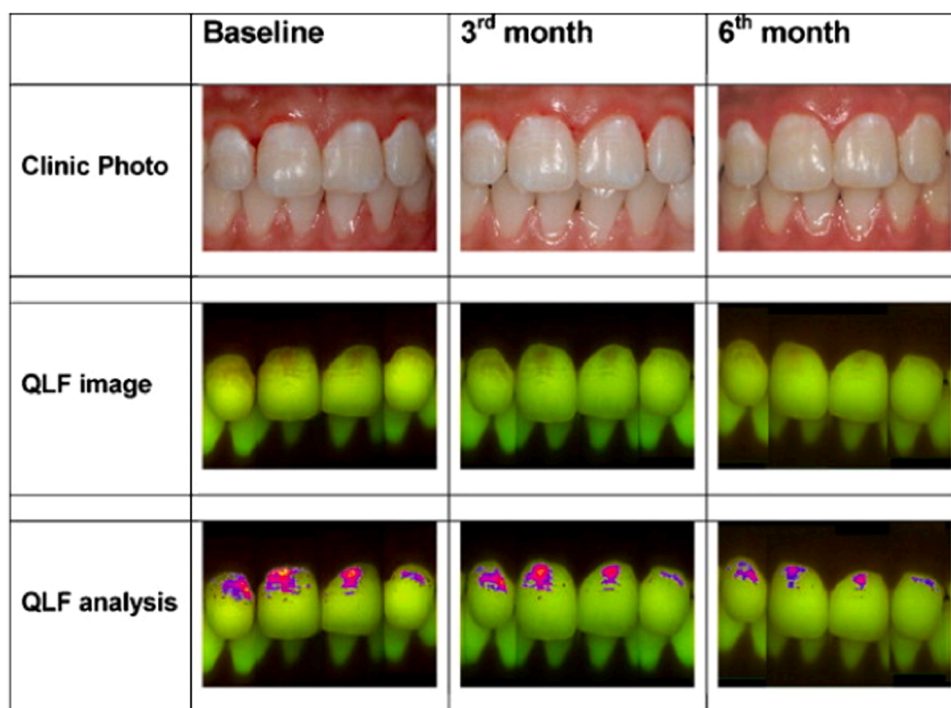


Fig. 5. Monitoring enamel remineralization using QLF. In QLF images, lesions appear as gray/dark areas. Through fluorescence intensity analysis, the extent of remineralization (blue-red scale) can be assessed. Reproduced from [48] - Creative Commons CC-BY-NC-ND.

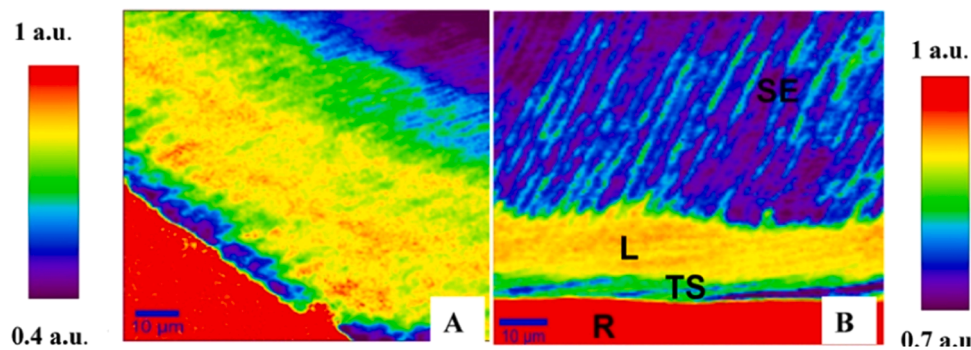


Fig. 6. FTIR mapping of (A) natural lesions and (B) chemically-induced caries. Images are constructed from the ratio between the intensity of the carbonate and phosphate vibration bands. Reproduced from [52] – Creative Commons CCBY.

peak of phosphates is broad but one of the amide vibration bands is only ca. 3 times less intense, allowing for a more detailed study of the organic matrix [51]. Therefore, Raman is more accurate to study the mineral component of tissues whereas FTIR provides more information on their organic content.

If successful, the remineralization reaction should yield to a mineral phase parent to hydroxyapatite of chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Therefore, determination of the weight or atomic ratio of Ca over P can serve as a first indicator of remineralization. Thus, the use of EDX to determine the Ca and P content, allowing for calculation of the Ca/P ratio, is by far the most popular method in this area [16]. EDX measurements are performed in similar conditions as SEM but, instead of recovering outgoing electrons, the EDX detector collects the X-Ray light emitted by atoms. Other elements, such as O, C and F, are also sometimes quantified by EDX [19]. Alternatively, the fluoride content can be obtained by electrochemical measurements after sample dissolution.

3.7.3. Surface morphology

Besides remineralization extent and chemical nature, one half of the considered studies investigated the surface morphology of the tissue after treatment. All of them used SEM imaging for this purpose. Additional quantitative data about surface topology were obtained using CSLM or AFM (Atomic Force Microscopy). In the first case, CSLM imaging is performed without staining so that only the autofluorescence of the sample is monitored [23]. As this fluorescence is very weak, only its surface is imaged. After 3D reconstruction, it is possible to determine surface roughness average, *i.e.* the average height difference between peaks or pits and the mean surface. AFM is a very powerful tool for surface analysis as it allows very high-resolution imaging. It consists in scanning a rigid probe with diameters 100 nm or below attached to a flexible cantilever very close to the surface of interest. When the probe meets a peak (or valley), the cantilever bends to permit its lift (or drop). This deflection, that is in most cases proportional to the height variation, can be optically-detected. Since the probe is very small and the cantilever very sensitive, imaging resolution is at the nm scale. Thus, it allows for both individual particle imaging and surface topology quantification [18].

3.7.4. Other miscellaneous techniques

Fluorescence measurements using the DIAGNodent® technique have also been used to evaluate remineralization [25]. This technique takes advantage of the production of fluorescent molecules by cariogenic bacteria and is therefore very well-suited for caries detection [53]. The fluorescence intensity is converted to a value according to the manufacturer scale. As for other fluorescence techniques, the recovered intensity should depend on the mineral density and, therefore, could in principle roughly reflect remineralization.

In the case of biologically-induced caries, it is expected that the

treatment also has some antibacterial effect and the fate of the seeded bacteria was therefore studied. On the one hand, live/dead fluorescent staining is used to visualize biofilm formation on the sample as well as bacteria viability using CSLM [31]. SEM after cell fixation is also used to investigate biofilm density and cell morphology [29]. On the other hand, biofilms can be detached from the samples and bacterial density determined using the agar plate counting technique [35]. Finally, when collagenase was added to the remineralization solution, collagen degradation was monitored by colorimetric titration of hydroxyproline, a specific by-product of collagen biodegradation [27].

3.8. Combination of techniques

From Table 1, it can be noticed that 20 over 24 of the studied papers involved techniques that belong to at least two of the above-defined categories, 11 spanned over at least three categories and only 1 used at least one technique in each category. Miscellaneous techniques were most often complementing two other categories. Mineralization extent was the most represented category (all but two studies) while chemical characterization and surface morphology were investigated in an almost equal number of works.

4. Discussion

The induction of artificial carious lesions in enamel and/or dentine is an important modeling tool to investigate strategies for their detection, prevention, or treatment, especially remineralization strategies. In this context, the aim of this systematic study was to identify which technique (s) allow for the most complete and accurate characterization of the demineralized/remineralized areas.

Our hypothesis is that besides spatial information (mainly lesion depth) and mineral density, it is necessary to characterize the chemical nature of the mineral formed following remineralization strategies. In contrast, a first information gained from this systematic review is that publications devoted to *in vitro* characterization of primary artificial caries in which at least three different analytical techniques were used are relatively few (24 over 225 eligible articles) and all but one of these are dedicated to remineralization studies.

Among these, many of them focus on surface characterization of the remineralized samples. The simpler approach combines SEM observation, EDX analysis of the Ca/P ratio and surface hardness measurement [16,20,30]. In many cases, SEM images are not analyzed in details and are sometimes even not shown in the article [13]. Coupling these observations with surface hardness measurements introduce some quantitative assessment of mineralization but this is mainly used to compare different remineralization conditions rather than to characterize the mineral phase *per se*. Complementation with EDX analysis offers some indication about the chemical nature of the mineral. In some studies, surface morphology is investigated in more details, using AFM or CSLM,

for example [18,19]. Because these techniques only investigate the sample surface, they do not allow for a full characterization of the remineralization process but can be useful as a preliminary qualitative evaluation of given protocols.

To check for the spatial extension of remineralization as well as to evaluate the density of the mineral phase, micro-CT must be preferred as it allows obtain 3D information [31,32]. It also has the benefit of being a non-destructive method. As alternatives, hardness measurements, TMR imaging or CSLM combined with dye impregnation performed on transversal slices provide reliable information [17,27], although they cannot assess the homogeneity of the remineralization over the whole caries volume. The two first methods can give access to mineral density if enamel samples are used as reference. The last one evaluates the non-mineralized volume fraction but, because it is based on the fluorescence of stained organic matter, whose measured intensity also depends on the mineral density, it cannot be fully quantitative. The same is true for other optical methods such as PS-OCT, FIRE and QLF [12,15]. Noticeably, SEM imaging of transversal slices can also be useful for both visualizing the demineralized/remineralized area and qualitatively assess mineral density [14].

However, taken alone, these techniques cannot ascertain that the deposited mineral phase is hydroxyapatite. Therefore, they must be combined with at least another technique. As mentioned earlier, the XRD spectra is an unambiguous signature of the chemical nature of the new mineral phase and also provide information about its crystallinity [14,21,27]. FTIR or Raman spectroscopy data may be more difficult to interpret but the former has the advantage of also monitoring the protein content of deposit [35]. Thus, combining XRD and one vibrational spectroscopy offer a quite complete chemical characterization of the remineralized area [31]. In contrast, EDX should be taken with caution, as it is very sensitive to the topology of the surface and has low sensitivity for light elements such as O and C.

To end up this section, it is worth pointing out the very small occurrence of biological characterizations of the lesions [29,31,35]. Indeed, investigation of the evolution of bacterial population after biologically-induced caries is of interest. However, when deep dentin lesions are considered, it is expected that both demineralization and remineralization strategies have an impact on dental cells. Thus, histological staining and/or proteomics analyses could be valuable tools for deeper understanding of the ongoing processes.

Taken together, the use of micro-CT for both 3D imaging and mineral density measurements, with FTIR and/or XRD constitutes, to our opinion, the most suitable combination of techniques to study demineralization/remineralization processes. When micro-CT is not available, the association of SEM, hardness measurements with FTIR and/or XRD for analysis of transversal slices (and not surface analysis only) of the sample appears as an insightful approach. If FTIR or XRD are not available, mapping Ca and P and determination of the Ca/P ratio by EDX provides some indication of the mineral composition.

5. Conclusion

In a clinical context, demineralization extent as induced by caries is mainly evaluated by its depth while remineralization strategies are evaluated by the reinforcement/recovery of the mechanical properties of the tissue. However, for a deeper study of these processes *in vitro* using artificial caries, characterization of the damaged/repared tissue must be more detailed and, in particular, include a 3D evaluation of the mineralization success and some chemical information about the newly-formed phase. The combination of micro-CT with vibrational spectroscopies and/or X-Ray diffraction appears as the best option for this purpose. As alternative to micro-CT, transverse SEM imaging and microhardness measurements are also particularly useful. The fact that several of these techniques are not clinically applicable and/or not of widespread availability, or even sufficient knowledge, in the dentistry community can explain why a very small fraction of papers identified

during this systematic review do provide a detailed assessment using a multi-technique approach. It is the hope of the authors that this review will contribute to bridge this gap for an improved development of caries remineralization strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dentre.2023.100073.

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