Synthesis, characterization and bioevaluation of partially stabilized cements for medical applications

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Abstract: Materials for dental applications, i.e., white mineral trioxide aggregate (WMTA) and partial stabilized cements (PSC) were obtained using the sol-gel method. The presence of ZnO or/and CaF₂ additions in the starting mixture induced changes in the composition, morphology and grindability of PSCs as compared with WMTA. The presence of foreign elements (Zn or F) in the crystalline lattice of mineralogical phases, increased their grindability. Thermal analysis (TG&DTA) was used to assess the kinetics of hydration process in binding systems based on WMTA/PSCs. The presence of foreign elements in PSCs systems increases the reactivity vs. water of these materials and consequently, the compressive strength developed after 28 days of hardening at 37°C are higher as compared with WMTA. The in vitro bioevaluation results (trypan blue staining, eukaryotic cells cycle assay by flowcytometry) accounted for a high biocompatibility of the obtained materials demonstrating their potential use for biomedical applications.

Keywords: Partial stabilized cement • Sol-gel synthesis • Hydration and hardening processes • Compressive strength • Cytotoxicity- eukaryotic cell cycle stages

1. Introduction

Mineral trioxide aggregate (MTA) is a biocement developed for surgical endodontic treatment, mainly for retrograde filling and perforation repair [1-5]. The main requirements for a material with such applications are: good sealing ability, quick setting, dimensional stability and biocompatibility [4-7]. MTA consists of mineralogical phases also present in portland clinker (PC), i.e., calcium silicates – Ca₃SiO₅ (C₃S), Ca₂SiO₄ (C₂S) and calcium aluminate – Ca₃Al₂O₆ (C₃A) [3,4,8]. The development of mechanical strength is determined in these systems mainly by C₃S and C₂S hydration, while the C₃A is responsible for cement setting [9,10]. Although the mineralogical composition of portland cement and MTA is similar, the substitution of MTA with PC is presently discouraged [4]. Two types of MTA were used for endodontic treatments, i.e., of gray (GMTA) and white (WMTA) colour [4,8,11]. The lighter colour of WMTA is mainly due to a lower content in colouring ions, such as iron, as well as its smaller particles size [4,11]. WMTA is preferred for dental restoration operations for aesthetical purposes.

Some studies reported a long setting time and poor handling properties of MTA [1,5,12], therefore a new material called Partial Stabilized Cement (PSC) has
been developed [5]. This material consists of a silicate cement in which transition elements (Co, Cr and Zn) are added in order to create crystal defects and high energy state which favours the decrease of the cement setting time.

The synthesis of C₃S, C₃S and C₃A via solid-state reactions consists in the sintering of stoichiometric mixtures of oxides or carbonates at high temperatures for long time [9,10]. Moreover, this type of synthesis implies multiple consecutive thermal treatments and intermediary material grinding; these operations could lead to introduction of material impurities and consequently to its colour and other properties change. Therefore the sol-gel method was used to synthesize C₃S [13,14], C₃A [15], GMTA [16] and WMTA [17,18]. The main advantages of this synthesis route (as compared with the conventional one) are lower thermal treatment temperatures and higher purity of the resulted products [13-18].

Preliminary results showed the possibility to synthesize WMTA with good in vitro biocompatibility by a sol gel route [18]. In order to obtain materials with high reactivity vs. water and consequently with a faster development of mechanical strength, two methods were studied: i) preparation of accelerated portland cement (APC) by WMTA mixing with CaCl₂ addition [19] and ii) synthesis by sol-gel route of PSCs with ZnO, CaF₂ or Zn+ CaF₂ mixtures. The choice of these two additions in PSC synthesis was based on the fact that Zn, in small quantities is generally harmless to human body and is a key element for bone development [5,17,20], while CaF₂ is a well known fluxing agent [21,22] and is a caries preventing agent [23,24].

In this paper we present the influence of ZnO or/and CaF₂ additions on the composition, morphology and grindability of PSCs synthesized by a sol gel route, as well as on the hydration and hardening processes and properties developed by these materials, including in vitro cytotoxicity and influence of the eukaryotic cell cycle.

2. Experimental procedure

2.1. Synthesis of WMTA and PSCs

One WMTA composition (K) and four PSC compositions (KZ, KZF and KF) – Table 1, were synthesized using the sol-gel route, previously described by Voicu et al. [18]. The advantage of this method, as compared with the one proposed by Wang et al. [16] is the use of a lower temperature thermal treatment (1350°C) of shorter duration (30 minutes).

The materials used for the sol-gel synthesis were: calcium nitrate (Ca(NO₃)₂·4H₂O, >99.0%, Reag. ACS., Sigma-Aldrich, Germany), aluminium nitrate (Al(NO₃)₃·9H₂O, >98.5%, Reag. ACS, Sigma-Aldrich, Germany), tetraethyl-orthosilicate (C₂H₅O₃Si, 99.0%, Reag. ACS., Sigma-Aldrich, Germany- TEOS), zinc nitrate (Zn(NO₃)₂·6H₂O, >98%, Reag. ACS., Sigma-Aldrich, Germany) and calcium fluoride (CaF₂, >99.9%, Reag. ACS., Sigma-Aldrich, Germany). These materials were dosed in order to obtain a stoichiometric mixture corresponding to the compositions presented in Table 1.

The main steps of the sol-gel synthesis were the following: calcium nitrate, followed by aluminium nitrate, zinc nitrate or/and calcium fluoride were dissolved in 180 mL water, under magnetic stirring, until a clear solution was obtained. TEOS was hydrolysed using a molar ratio TEOS: water of 1:4. The two solutions were mixed and continuously stirred at 60°C for 4 hours, and then kept the next 96 hours at 70°C, to facilitate the water evaporation and to accelerate the polycondensation reaction, resulting in the formation of a viscous gel. This gel was then dried at 120°C for 420 hours and the final product was a white powder [18].

The powders were pressed in pellets and thermally treated at 1350°C, for 30 minutes. Rapid cooling was performed in air.

In order to obtain the cements these materials were grind for two hours in a laboratory planetary mill.

2.2. Materials analysis

The white powders resulted by gel drying were analysed by thermal analysis (DTA/TG/DTG) and by X-ray diffraction analysis (XRD). Thermal analysis was performed using a Shimadzu DTG-TA-60, in the 20-1000°C temperature range, with a heating rate of 10°C min⁻¹, in air. X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer, with Ni-filtered CuKα radiation (λ=1.5406 Å), with scan step of 0.02° and counting time of 0.6 s step⁻¹.

The elemental composition of materials (WMTA and PSCs) obtained from the thermal treatment was assessed by X ray fluorescence using a Rigaku ZSX Primus XRF machine. The material’s compositions presented in Table 2 are similar to those calculated based on the designed mineralogical composition (Table 1).

The mineralogical composition was assessed by XRD and their morphology by scanning electron microscopy (SEM) using a HITACHI S2600N equipment. The specimens for SEM analysis were covered with a thin silver layer deposited by dc-sputtering.
The granulometric characteristics (i.e., Rosin-Ramler-Sperling diagrams) of the powders resulted after two hours of grinding in the laboratory planetary mill were assessed by laser granulometry by means of a Malvern Mastersizer 2000 laser granulometer.

Free lime content of materials thermally treated at 1350°C for 30 minutes was assessed by EDTA method as prescribed in European and corresponding national norm - SR EN 196-2:2006 [25].

The hydration and hardening processes of WMTA/PSCs were assessed by XRD and thermal analysis of pastes (solid/water ratio was 1 g per 0.33 mL), after 28 days of curing. The hydration was stopped by acetone washing and drying at 60°C for 10 hours.

The compressive strength was assessed on paste specimens - cylinders (φ=10 mm and h=10 mm), cured the first day in a mould at 37°C and after demoulding submerged, up to 28 days, in distilled water at 37°C.

### 2.3. Biological assays

#### 2.3.1. Cells

HCT8 line (ECAC90032006) and human diploid cells (HDC) were used in our experiments. HCT8 line was cultivated in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated bovine serum and penicillin/streptomycin at 37°C with 5% CO₂. Diploid fibroblasts were cultivated in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated bovine serum and penicillin/streptomycin at 37°C with 5% CO₂.

#### 2.3.2. Cytotoxicity Assay

Cytotoxicity was performed using Trypan blue staining after the treatment with 100 µg mL of the tested cements. In brief, a freshly prepared solution of 50 µL Trypan blue (0.05%) in distilled water was mixed with 50 µL of each of the cellular suspension during 5 min, spread

### Table 1. Designed mineralogical composition of WMTA and PSCs.

| Material | 3CaO·SiO₂ (C₃S) (%) | 2CaO·SiO₂ (C₂S)(%) | 3CaO·Al₂O₃ (C₃A) (%) | ZnO (%) | CaF₂ (%) |
|----------|---------------------|---------------------|----------------------|---------|----------|
| WMTA K   | 70                  | 24                  | 6                    | -       | -        |
| KZ       | 70                  | 24                  | 6                    | 1       | -        |
| PSCs KZF | 70                  | 24                  | 6                    | 1       | 0.5      |
| KF       | 70                  | 24                  | 6                    | -       | 0.5      |

### Table 2. Elemental compositions of synthesised biocements.

| Composition (%wt.) | Clinker | K | KZ | KZF | KF |
|--------------------|---------|---|----|-----|----|
| Calculated         |         |   |    |     |    |
| F                  | 0       | 0 | 0  | 0.24| 0.24|
| Al                 | 1.20    | 1.19| 1.19| 1.20|
| Si                 | 12.50   | 12.38| 12.31| 12.44|
| Ca                 | 50.68   | 50.17| 50.18| 50.68|
| Zn                 | 0       | 0.80| 0.80| 0   |
| O                  | 35.62   | 35.47| 35.29| 35.45|
| Assessed by XRF    |         |   |    |     |    |
| F                  | 0       | 0 | 0  | 0.24| 0.24|
| Al                 | 1.18    | 1.17| 1.18| 1.17|
| Si                 | 12.47   | 12.37| 12.27| 12.42|
| Ca                 | 50.66   | 50.15| 50.17| 50.66|
| Zn                 | 0       | 0.79| 0.80| 0   |
| O                  | 35.69   | 35.52| 35.34| 35.51|
onto a microscope slide and covered with a coverslip. Nonviable cells became blue-stained. At least 200 cells were counted per treatment \[26,27\].

2.3.3. Cell cycle

For cell cycle analysis, HDC or HCT8 cell lines were treated with 100 µg cement mL\(^{-1}\), and maintained for 24 h at 37°C, 5%CO\(_2\) and in humid condition. Thereafter, cells were harvested, washed in phosphate saline buffer (PBS, pH 7.5), fixed in 70% cold ethanol and maintained at -20°C, overnight. Each sample was washed in PBS, treated with 100 µg mL\(^{-1}\) ribonuclease A (RNase A) for 15 minutes and coloured with 10 µg mL\(^{-1}\) propidium iodide by 1 hour incubation at 37°C. After cell staining with propidium iodide, the acquisition was done using Epics Beckman Coulter flowcytometer. Data were analysed using WinMDI 2.9 software and expressed as fractions of cells in the different cell cycle phases \[28,29\].

3. Results and discussion

DTA, TG and DTG curves of the white powders resulting from gel drying are presented in Figs. 1 and 2.

On the DTG curve of WMTA (K), are present the following processes, correlated with the information provided by DTA curve: calcium and aluminium nitrates dehydration and decomposition – effect with maximum at 331°C, followed by calcium hydroxide decomposition – effect with maximum at 497°C, anhydrous Ca(NO\(_3\))\(_2\) melting (effect with maximum at 564°C) and finally the effect from 849°C attributed to the decomposition of calcium carbonate, formed by partial carbonation of calcium hydroxide \[9,10,30,31\].

The DTA curves of KZ and KF (Fig. 2a) present similar effects in the range of 400-700°C. A higher weight loss recorded in this temperature range (see also TG – Fig. 2b) for the specimen KZ suggests the presence of higher amount of calcium nitrates.

The calcium nitrates are formed in these compositions due to the high concentration of NO\(_3^-\) in the starting solution; consequently partial re-precipitation of calcium nitrates could occur. The presence of calcium nitrates (Ca(NO\(_3\))\(_2\) – JCPDS [07-0204] and Ca(NO\(_3\))\(_2\)•2H\(_2\)O - JCPDS [27-0087]) in the dry gels is confirmed also by the XRD spectra- Fig. 3. CaCO\(_3\) assessed on DTA curves cannot be identified with certainty on the XRD spectra, most probably due to its low quantity (under XRD detection limit).

The free lime values assessed on WMTA and PSCs obtained by thermal treatment at 1350°C for 30 minutes are presented in Table 3. Accepted free lime values are not greater than 2% as it adversely affects the quality of cement \[9\]; as it can be seen from Table 3, all values are well below this limit.

The XRD spectra of WMTA and PSCs are presented in Fig. 4. The main mineralogical compounds formed by thermal treatment are: calcium silicates (3CaO•SiO\(_2\) - JCPDS [42-0551], JCPDS [76-0623], JCPDS [31-0301]...
and 2CaO·SiO₂ - JCPDS [33-0302], JCPDS [33-0303], JCPDS [70-0388]) and calcium aluminate (3CaO·Al₂O₃ - JCPDS [38-1429], JCPDS [32-0150]).

The XRD peak in the window 2θ = 51-53° (Figs. 4 and 5) can provide information about 3CaO·SiO₂ (C₃S) polymorphism [9] as well as the presence of foreign ions in its crystalline structure. The shifting of the C₃S peak at 2θ = 51.86° (CuKα radiation) - recorded for specimen K, to lower values for the PSCs (i.e., 51.72° - KZ, 51.76° - KZF and 51.8° - KF) suggests the presence of foreign ions in the C₃S crystal lattice [22]. The incorporation of foreign ions determines also the increase of the crystallite size. Table 3 presents the average size of C₃S crystallites calculated with Debye-Scherrer equation [32] i.e., evaluated from the line broadening of 29.5(29.6)°, 30.7(30.8)° and 51.7(51.8)° peaks.

The morphology of WMTA and PSCs was assessed using scanning electron microscopy (SEM) – Fig. 6.

As it can be seen on Figs. 6a and 6b in SEM micrographs of K specimen, polyhedral C₃S crystals (average size 6-8 μm) and round C₂S crystals (average size 2-6 μm) were identified. In case of the specimen with ZnO addition (KZ), the SEM micrographs (Figs. 6c and 6d) showed agglomerations of small crystals of 3-6 μm average size. For the specimens with CaF₂, the amount of liquid (molten) phase increased (Figs. 6e-6h) as compared with K and KZ specimens. These results are in good correlation with other literature data, fluorides being well known as mineralizers and fluxing agents [22,30].

The influence of ZnO and/or CaF₂ additions on the grindability of obtained clinkers (WMTA and PSCs) can be estimated considering the granulometric characteristics of the powders formed after 30 minutes of grinding, in similar conditions (Fig. 7). The particle sizes of PSCs (KZ, KZF and KF) are smaller than those of WMTA (K), i.e., the grindability of these materials is higher. This can

Table 3. Characteristics and properties of synthesised biocements.

| Specimen | K | KZ | KZF | KF |
|----------|---|----|-----|----|
| Free lime* (% | 1.05 | 0.32 | 0.28 | 0.41 |
| Average C₃S crystallite size** - d (nm) | 24.54 | 35.38 | 38.51 | 28.91 |
| Compressive strength** - Cₛ (MPa) | 8.57±0.42 | 13.39±0.46 | 14.44±0.56 | 13.76±0.57 |

* assessed on materials thermally treated at 1350°C for 30 minutes; ** assessed on cement pastes hardened for 28 days at 37°C
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be explained by the incorporation of foreign elements in the silicates and aluminate structures, thus affecting the microsymmetry and electrostatic relations, the chemical bonds or/and coordination of ions in the structure [33]. The increase of grindability is important in connection with both material reactivity vs. water as well as its aesthetical properties – the degree of whiteness of the material increases with the increase of its fineness.

The hydration and hardening processes in WMTA and PSCs systems were assessed by XRD and DTA/TG analysis on pastes hydrated for 28 days. The XRD spectra presented in Fig. 8 show the presence of crystalline anhydrous phases (C$_3$S, C$_2$S and C$_3$A), as well as of portlandite (calcium hydroxide), the main crystalline product formed by calcium silicates hydration. The small amount of calcium carbonate present in the pastes hydrated for 28 days is formed by portlandite carbonation, its sensitivity to CO$_2$ being well known [9,30].

The thermal analysis can provide quantitative information regarding both crystalline hydrates (portlandite and calcium aluminates hydrates) as well as poorly crystalline hydrates such as calcium silicates hydrates (C-S-H) [9,10,30]. The DTA and TG curves

Figure 4. The XRD spectra of materials obtained by thermal treatment at 1350°C for 30 minutes.

Figure 5. C$_3$S peaks in the XRD window 2$\theta$ = 51-53°.
Figure 6. SEM micrographs of: K - a and b; KZ – c and d; KZF – e and f; KF – g and h.
Figure 7. Rosin-Rammler-Sperling diagram of materials grinded for 30 minutes in similar conditions: a- K; b-KZF; c-KZ; d-KF.

Figure 8. XRD spectra of pastes hydrated 28 days.

Figure 9. DTA (a) and TG (b) curves of K and KZ pastes hydrated 28 days.
recorded on pastes hydrated for 28 days are presented in Figs. 9 and 10. Three endo-effects could be seen on DTA curve:

- the large effect from 70-82°C and the shoulder from 142-164°C are most probably due to the superposition of the following processes: loss of humidity and water bound in calcium silicates hydrates (C-S-H) and calcium aluminate hydrates;
- the endo-effect from 455-487°C is due to the portlandite dehydration, and
- the large endo-effect from 657-685°C (and the shoulder at 856-852°C) is attributed to the decomposition of CaCO₃, with different crystallinity degrees, formed by Ca(OH)₂ carbonation during specimens preparation and curing.

The results presented in Table 4 for the PSCs (KZ, KZF and KF), show that the weight loss values recorded between 20-1000°C are higher when compared with WMTA (K specimen). The higher reactivity vs. water of PSCs, as compared with WMTA, is the direct consequence of the modification of crystalline structure, morphology and grindability induced by the ZnO and CaF₂ additions.

The compressive strength of PSCs specimens are higher than those recorded for WMTA (see Table 3), which is in good agreement with the information obtained by DTA&TG.

The dye exclusion test was used to determine the number of viable cells present in a cell suspension after 24 hours treatment with the obtained cements (pastes hydrated 3 and 28 days). This test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium iodide, whereas dead cells do not, so that these substances accumulate inside the cells [31].

The cell cytotoxicity was calculated from the tripan blue assay on two cellular cell lines, i.e., HCT8 and DHC. The cytotoxicity induced by the tested compounds on the two cellular lines was reduced, ranging from 7%
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No significant difference was observed between the 3 and 28 days hydrated pastes.

The eukaryotic cell cycle consists of two basic stages: interphase and mitosis (M). The interphase has three stages called Gap 1 (G1), Synthesis (S), and Gap 2 (G2). In the G1 phase, the cell is growing, replicating cytoplasmic organelles and preparing for replication of its DNA, which is synthesized during S phase. During the G2 phase cells are preparing for division, which occurs in the M phase of the eukaryotic cell cycle. The influence of different substances on the eukaryotic cell faith might be cell cycle phase-dependent, which is a reason why this test is used for the in vitro assessment of the cytotoxicity.

With respect to the influence of the tested substances on the cellular cycle of the two lines, no significant changes were observed in the two tested cellular lines on the pastes hydrated for 3 days (Fig. 11) and for 28 days (Fig. 12).

### 4. Conclusions

The results presented in this paper show that white mineral trioxide aggregate (WMTA) and partial stabilized cements (PSC) can be synthesized by sol-gel method. The presence of ZnO or and CaF₂ additions in the starting mixture determines the modification of composition, morphology and grindability of PSCs as compared with WMTA.

For PSCs, the increase of C₃S crystalite size and the shifting of its XRD peaks (as compared with WMTA), suggests the presence of foreign elements (Zn or and F) in the C₃S crystal lattice with positive influence on the materials grindability; the increase of grindability is important in connection with both material reactivity vs. water as well as its aesthetical properties. The degree of whiteness increases with the increase of fineness.

Thermal analysis (TG&DTA) showed an increase of the hydration rate in the binding systems with PSCs (as compared with WMTA) with direct consequences on the mechanical strength of synthesised materials. Compressive strength increases of 55-68% were recorded after 28 days of hardening at 37°C for the studied PSCs as compared with WMTA.

The in vitro bioassays results demonstrate a high biocompatibility of the obtained materials demonstrating their potential for biomedical applications.

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**Table 4.** Weight losses recorded on TG curves and calculated Ca(OH)₂ content for pastes hydrated for 28 days.

| Specimen | Weight loss in temperature (°C) range: | Ca(OH)₂ content (%) | Corrected Ca(OH)₂ content (%) |
|----------|----------------------------------------|----------------------|-------------------------------|
|          | 20-1000 | 400-575 | 575-1000 | | |
| K        | 20.27   | 5.60    | 5.61    | 23.02 | 32.45 |
| KZ       | 22.51   | 6.21    | 5.98    | 25.53 | 35.58 |
| KZF      | 23.48   | 6.29    | 7.04    | 25.86 | 37.70 |
| KF       | 22.32   | 6.45    | 6.52    | 26.52 | 37.48 |

* calculated based on the weight loss recorded between 400-575°C; ** calculated considering also Ca(OH)₂ converted in CaCO₃ (based on weight loss between 575-1000°C)

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**Figure 12.** Influence of WMTA (K) and PSC (KZ, KZF and KF) pastes hydrated for 28 days on the cellular cycle phases of: a – DHC; b- HCT 8 cells.
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