Materials Science & Engineering C 109 (2020) 110592

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

The synergistic effects of SrF_2 nanoparticles, YSZ nanoparticles, and poly-ε-L-lysin on physicomechanical, ion release, and antibacterial-cellular behavior of the flowable dental composites

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ARTICLE INFO

Keywords:
SrF_2 nanoparticles
YSZ nanoparticles
Poly-ε-L-lysin
Pit-and-fissure sealant
Antibacterial property
Fluoride release

ABSTRACT

Resin-based pit-and-fissure sealants (flowable resin composites) were formulated using bisphenol-A-glycerolatedimethacrylate (Bis-GMA)-triethylene glycol dimethacrylate-(TEGDMA)-diurethanedimethacrylate (UDMA) mixed monomers and multiple fillers, including synthetic strontium fluoride (SrF_2) nanoparticles as a fluoride-releasing and antibacterial agent, yttria-stabilized zirconia (YSZ) nanoparticles as an auxiliary filler, and poly-ε-L-lysin (ε-PL) as an auxiliary antibacterial agent. Based on the physical, mechanical and initial antibacterial properties, the formulated nano-sealant containing 5 wt% SrF_2, 5 wt% YSZ and 0.5 wt% ε-PL was selected as the optimal specimen and examined for ion release and cytotoxicity. The results showed an average release rate of 0.87 $\mu g \cdot cm^{-2} \cdot day^{-1}$ in the aqueous medium (pH 6.9) and 1.58 $\mu g \cdot cm^{-2} \cdot day^{-1}$ in acidic medium (pH 4.0). The maximum cytotoxicity of 20% toward human bone marrow mesenchymal stem cells (hMSCs) was observed according to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cytotoxicity assay and acridine orange staining test. A synergy between SrF_2 nanoparticles and ε-PL exhibited a better antibacterial activity in terms of colony reduction compared to the other samples. However, the inclusion of SrF_2 and ε-PL caused mechanically weakening of the sealants that was partly compensated by incorporation of YSZ nanoparticles (up to 10 wt%).

1. Introduction

Dental caries (known as tooth decay) or cavities are irreversible microbial diseases affecting the calcified tissues of the teeth that are characterized by demineralization of the inorganic fraction and decomposition of the organic matrix of tooth leading to the cavity formation [1]. Dental caries are still one of the most common illnesses in the world and the most common disease among all chronic oral and dental illnesses. Nearly two in five of the world's population suffers from caries in their permanent teeth. Depending on the geography and lifestyle, the prevalence of dental caries or untreated caries among children aged 6–11, adolescents and youths aged 12–19, as well as people aged 20 and over, is estimated to be 21%, 58%, and 91%, respectively [2]. The presence of bacterial species responsible for declining the salivary pH, lack of proper oral hygiene, diet type, and tooth microstructure are among the most important factors predisposing to the onset of tooth decay. Decay-generating bacteria, such as oral *streptococci*, especially the *mutans* group and lactic acid-producing bacteria play an important role in the pathogenesis of dental caries [3]. *Streptococcus mutans* bacteria are gram-positive prokaryotes that are believed to be the main causative agents for the tooth decay through accumulation on dental enamel, plaque formation and localized demineralization of dental enamel. Uncontrolled increase in bacteria leads to dentin penetration and pulp infection, which causes severe pain, pulp necrosis, tooth loss, and systemic infections [4]. In addition, *Enterococcus faecalis* as another gram-positive bacteria, is an opportunistic pathogen and one of the leading causes of nosocomial infections [5].

The common methods of tooth decay prevention include: (i) the systemic approach through the use of fluoride compounds and antidecay sweeteners such as xylitol, saccharin and aspartame; and (ii) the topical method for remineralization amplification, demineralization inhibition, and stopping glycolysis in oral microbes using sealants and fluoride therapy [6,7].

Most of dental caries and abnormalities occur, especially in the microcracks and grooves on posterior teeth, which refer to as pits and fissures. Pits and fissures are ideal sites for housing bacteria and plaque accumulation [8, 9]. To deal with caries under such conditions, the use of protective materials which seal pits and fissures, has shown to be

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https://doi.org/10.1016/j.msec.2019.110592
Received 25 February 2019; Received in revised form 15 December 2019; Accepted 21 December 2019
Available online 28 December 2019
0928-4931/

incredibly effective in preventing tooth decay [10,11]. From the past to the present day, various materials have been used to seal the dental grooves and cavities, including silver nitrate, amalgam, cyanoacrylate, polyurethane, glass-ionomer cement, and resin composites. Owing to their ease of use, high durability, proper mechanical strength, and favorable tooth-colored appearance, composite resins are the most common and reliable materials for sealing dental abnormalities [12,13].

Although a wide range of materials are used as sealants [14], the material most suitable for the indication of pit and fissure sealing is still under question particularly, in terms of the occurrence of secondary caries at the interface of the tooth and restorative materials. It causes the chemical and mechanical destruction of the restorative materials [15,16]. Secondary or recurrent caries referred to dental lesions on the margins of existing restorations that themselves undergo caries [16]. Resin-based sealants and glass ionomer sealants are most commonly used as pit and fissure sealing materials [17]. Numerous investigations have reported on the significant benefits for resin-based sealants [18–21]. Owing to their ease of use, high durability, proper mechanical strength, and favorable tooth-colored appearance, composite resins are the most common and reliable materials for sealing dental abnormalities [12,13]. The most prominent advantage of them is their proper durability [22]. The adhesion property is another important factor for resin composites and membrane devices [23–25]. Direct restorative materials such as sealants are derivations of composite resins, as are dentin adhesives and orthodontic adhesives. The quality and stability of adhesive interfaces in enamel and/or dentin play a major role in the long-term clinical success of composite resin restorations [26]. Furthermore, another concern is the long term stability of restorative materials as a significant deterioration of the properties can intensely compromise the survival rate of restorative materials such as resin composites [21]. Thus, physicochemical stability is necessary for these products in order to possess an acceptable longevity. Among the most significant factors contributing to the failure of composite resins over time are the loss of brightness, dental stains, marginal infiltration, recurrent decay, dental wear, and fractures [27,28]. Resin-based sealants also have disadvantages when they are used as sealing materials. In terms of resin-based materials for sealing, one disadvantage includes polymerization shrinkage, potentially resulting in microleakage, which allows saliva and bacteria to penetrate the occlusal barrier [29,30]. Furthermore, a stronger biofilm accumulation seems to occur on resin-based materials [31]. Moreover, one of the latest innovations in the field of composite resins is the use of nanotechnology. Adding nanoparticles to composite resins could allow the production of flowable materials with better mechanical properties and flowability than previous sealants [14].

The high surface area of nanomaterials gives them superior properties that can be used to develop therapeutic and restorative therapies [11,32]. Meanwhile, metal fluoride nanoparticles (like SrF_2) have shown to play an effective role against oral bacteria. They can prevent decay due to their prolonged fluoride release, hence using fluoride-releasing nanoparticles in dental composite resins has attracted a great deal of attention toward preventing the secondary caries occurrence [33].

According to abovementioned explanations, it should be mentioned that, to our knowledge, no studies have been done on the synthesis of novel resin-based pit-and-fissure sealants based on composition of Bis-GMA–TEGDMA–UDMA mixed monomers, synthetic SrF_2 nanoparticles as a fluoride-releasing and antimicrobial agent, as well as YSZ auxiliary filler and poly-ε-L-lysine (ε-PL) as an auxiliary antimicrobial agent. In this research, we tried to evaluate the effect of SrF_2, YSZ and ε-PL additives on physicomechanical and antibacterial properties of Bis-GMA–TEGDMA–UDMA as the untreated resin-based pit-and-fissure sealants (control) through proper analytical techniques. Herein, we have hypothesized that SrF_2 nanoparticles and poly-ε-L-lysine could interact in a positive manner, leading to improved antibacterial activity of fissure sealants and the probable mechanical drawbacks thereof can be compensated by adding a proper amount of YSZ nanoparticles to sealants.

2. Experimental

2.1. Synthesis of SrF_2 nanoparticles

SrF_2 nanoparticles were synthesized according to the method in our recent paper [34]. Briefly, 100 ml deep eutectic solvent (DES) was prepared by mixing choline chloride ($C_5H_{14}ClNO$, Merck, Art. No. 500117) and strontium chloride ($Sr(Cl)_2.6H_2O$, Merck, Art. No. 107865) in 1:2 M ratio as well as water (5 wt%) on a magnetic stirrer at 80 °C. 2 g ammonium fluoride (NH_4F, Merck, Art. No. 101164) was added to DES and stirred for 2 h at the same temperature. Afterward, the precipitated powders were centrifuged, washed 5 times with deionized water, and eventually dried in a vacuum oven at 60 °C for 12 h.

2.2. Surface-silanization of SrF_2 and YSZ nanoparticles

The YSZ nanoparticles (Y_2O_3 [3.0 mol%]-ZrO_2, Sigma-Aldrich, Art. No. 572322) or synthetic SrF_2nanoparticles (5 wt%) were introduced into 100 ml n-hexane (C_6H_{14}, Merck, Art. No. 107023) containing 2 wt % propylamine (C_3H_9N, Sigma-Aldrich, Art. No. 109819) and 3 wt% 3-(Trimethoxysilyl)propyl methacrylate (γ-MPS, Sigma-Aldrich, Art. No. 440159). The suspension was stirred at 60 °C for 1 h. Thereafter, the solvent was removed in a rotary evaporator at 90 °C and the resulting powders were dried in a vacuum oven at 80 °C for 24 h.

2.3. Preparation of nanocomposite sealants

The sealants were formulated using bisphenol-A-glycerolatedimethacrylate (Bis-GMA, Sigma Aldrich, Art. No. 494356), triethylene glycol dimethacrylate (TEGDMA, Sigma-Aldrich, Art. No. 261548), and diurethanedimethacrylate (UDMA, Sigma-Aldrich, Art. No. 436909) monomers, camphorquinone (CQ, Sigma-Aldrich, Art. No. 124893) photoinitiator and 2-(dimethylamino) ethyl methacrylate (DMAEMA, Sigma-Aldrich, Art. No. 234907) auxiliary activator as well as synthetic SrF_2 nanoparticles, YSZ nanoparticles, and poly-ε-L-lysine ($(C_6H_{12}N_2O.HCl)_n$, Carbosynth, Art. No. FP14985) antibacterial additive according to the weight percent values given in Table 1. Firstly, CQ and DMAEMA were dissolved in the monomer mixture using a laboratory vacuum blender. Subsequently, SrF_2 and YSZ nanoparticles were added to the monomers according to the desired weight percentages and mixed for 1.5 h until a completely homogeneous mixture was obtained. In the end, poly-ε-L-lysine was added to the resulting pastes and dispersed for 20 min.

Table 1
Formulations of nanocomposite sealants.

Sealant	Bis-GMA-UDMA-TEGDMA [88–6-6] (wt %)	CQ-DMAEMA (wt%)	SrF_2 (wt %)	YSZ (wt%)	ε-PL (wt%)
F	99.0	1.0	0.0	0.0	0.0
Fε	98.5	1.0	0.0	0.0	0.5
FS10	89.0	1.0	10.0	0.0	0.0
FεS10	88.5	1.0	10.0	0.0	0.5
FY10	89.0	1.0	0.0	10.0	0.0
FεY10	88.0	1.0	0.0	10.0	0.5
FSY5	89.0	1.0	5.0	5.0	0.0
FεSY5	88.5	1.0	5.0	5.0	0.5
FS20	79.0	1.0	20.0	0.0	0.0
FεS20	78.0	1.0	20.0	0.0	0.5
FSY10	79.0	1.0	10.0	10.0	0.0
FεSY10	78.5	1.0	10.0	10.0	0.0

2.4. Mechanical properties of nanocomposites

Three-point flexural strength (FS) and flexural elastic modulus (E_f) were measured in accordance with ISO-4049. For this purpose, the pastes were introduced into a stainless steel mold of 25 × 2 × 2 mm between two glass slides and pressurized to extrude excess materials. The molded materials were then light-cured using an LED curing device (Woodpecker Light Cure Unit LED-D, output: 850–1000 mW/cm^2) and placed in a water bath at 37 °C for 60 min. The specimens were separated from the mold and incubated in distilled water at 37 °Cfor 24 h. The flexural strength was measured using Santam-DBBP-2 T machine according to the following equation [35]:

$$\mathrm{FS\ (MPa)} = \frac{3P_{max}L}{2bh^2} \quad (1)$$

in which, P_{max} is the maximum load(N), L is the distance between the supports, b is the width and h is the thickness of the sample.

The flexural elastic modulus was calculated using the equation [35]:

$$E_f = \left(\frac{P_{max}}{d}\right)\left(\frac{L^3}{[4bh^3]}\right) \quad (2)$$

where d stands for the deflection of the load-displacement curve in the linear elastic region.

For compressive strength (CS) measurement, the sealants were molded into cylindrical molds with 4 mm in diameter and a height of 6 mm. After light-curing, the specimens were placed in a water bath at 37 °C for 60 min. The mold was separated and the cured sealants were stored in distilled water at 37 °C for 24 h. The strength measurements were performed on a Santam-DBBP-2 T machine according to the equation [36]:

$$\mathrm{CS\ (MPa)} = \frac{4F}{\pi D^2} \quad (3)$$

where D is the diameter of the sample in mm and F is the maximum applied force in N.

2.5. Microstructure

To study the microstructure of the sealants, the fracture surface of the specimens were imaged using a Tescan Mira 3 LMU (working voltage 2 kV) field emission scanning electron microscope (FESEM) equipped with a Quantax 200 (Bruker) energy-dispersive X-ray spectroscope (EDS).

2.6. Fluoride release analysis

The release of fluoride (F^-) from the formulated sealants was investigated over a 100-day period in deionized water (pH 6.9) and acetic acid solution (pH 4.0). To this end, the disk-shaped sealants with dimensions of 15 × 1 mm were immersed in 25 ml of the solution and kept at 37 °C. At each time up to 100 days, the specimens were removed and immersed into a fresh solution. The old solution was analyzed for fluoride concentration. Fluoride concentration was measured using a fluoride ion-selective electrode (Fluoride ISE Metrohm). For measurement, 3 ml of the solution was mixed with 3 ml of total ionic strength adjustment buffer (TISAB: 58 g NaCl, 57 ml glacial acetic acid, 150 ml 6 M NaOH in 1000 ml deionized water) to minimize the ionic interferences that caused the measurement to be erroneous by increasing the ionic strength of the solution.

2.7. Polymerization shrinkage

In accordance with ISO-3521, the density (ρ) values of raw and cured sealants measured by pycnometer were utilized for the calculation of polymerization based on the following Eq. [37]:

$$\mathrm{Shrinkage\ }\Delta V\% = \left(1 - \frac{\rho_{uncured}}{\rho_{cured}}\right) \times 100\% \quad (4)$$

2.8. Solubility and water sorption

Five sealant specimens were molded as disks of 1.0 mm in thickness and 15 mm in diameter and light-cured for 30 s. In accordance with the method described in ISO 4049, the specimens were immersed in at least 10 ml of deionized water at 37 °C for 7 days. Based on the initial volume of the specimens (V), their initial mass before immersion (m_1), the mass after immersion in water (m_2), and the dry mass in the desiccator (m_3), the solubility and water sorption were calculated according to the following relations [37]:

$$\mathrm{Solubility\ (\mu g{\cdot}mm^{-3})} = \frac{m_1 - m_3}{V} \times 100 \quad (5)$$

and

$$\mathit{Water}\ \mathrm{sorption\ (\mu g{\cdot}mm^{-3})} = \frac{m_2 - m_3}{V} \times 100 \quad (6)$$

2.9. Antibacterial activity

Microorganisms play important roles in the onset and development of dental caries. Therefore, for initial evaluation of antibacterial activity, two kinds of bacteria including Streptococcus mutans (S. mutans: IBRC-M 10682) and Enterococcus faecali (ATCC 29212) which are known as the main gram-positive microbial agents of dental caries and the leading cause of resin-based composite failure were selected [38,39]. The latter oral microorganism is frequently associated with endodontic infections [40]. It is worth to mention that although gram-negative anaerobic bacteria have been shown to associate with periodontal diseases, Christian Splieth et al. found that anaerobic gram-negative bacteria associated with periodontal diseases were predominant in secondary caries in composite fillings [41]. The bacteria were tested according to the standard method in ISO 22196 as well as our previous work [42,43]. In brief, all specimens were sterilized by 70% ethanol in water and were exposed to UV radiation for 30 min. The specimens were measured for the colony forming units (CFUs) after incubation for 24 h. The specimens were placed in a separate well within a sterile 16-wells plate. *E. faecalis*, and S. mutans were transferred from the stock culture to the slant culture medium and incubated at 37 °C overnight. The bacteria suspension diluted to obtain a bacterial concentration of 10^6 CFUs/ml. Before incubating the discs anaerobically, the discs were completely washed with sterile phosphate buffered saline (PBS). The number of colonies was counted using Sana SL-902 colony counter.

The number of viable bacteria was determined according to the following equation:

$$N = (C \times D)/A \quad (7)$$

where N is the number of viable bacteria regained per cm2 per test specimen; C is the plate count; D is the dilution factor; A is the surface area of test specimen in cm^2.

2.10. Cellular behavior

Adequate contact between cells and test material is very important in biological evaluation of materials. Contact between cells and material can be achieved in three ways: direct contact, indirect contact, and contact through extracts [44–51]. Extraction technique with different extraction media has been frequently used in the cytotoxicity evaluation of different dental materials such as restorative materials [52,53], dental cements [54], amalgams [55], denture base resins [56], and dentine adhesives [57]. Therefore, for initial evaluation, we selected the short term cytotoxicity according to the other studies [20,58–60]. In

this research, Mesenchymal stem cells isolated from human bone marrow (hMSCs) were used to evaluate the cellular responses of the formulated sealants by MTT cytotoxicity assay and acridine orange staining test. The sealants were molded in discs with 4 mm in diameter and thickness of 2 mm, light-cured for 40 s, and incubated at 37 °C for 1 h. The specimens were crushed, ground, weighed and immersed in DMEM-containing cell culture plates, then incubated at 37 °C, 5% CO_2 for 24 h. The sealant extracts were passed through a filter (20 μm) and applied to the cultured hMSCs. The remaining steps proceeded in accordance with the recipe declared in our recent papers, based on the ISO 10993-12 specification [34,61,62].

2.11. Statistical analysis and data analysis

Each test was performed on five specimens. The significance of the results was evaluated by SPSS software (version 17) using one-way analysis of variance (ANOVA) statistical method. Herein, a p-value ≤ 0.05between two groups of data was considered as the significant discrepancy.

3. Results and discussion

3.1. Physico-mechanical properties of the formulated sealants

Fig. 1 shows the values recorded for the flexural strength, flexural elastic modulus and compressive strength of the formulated sealants. According to the results, the FY10 exhibits the best performance among the sealants in terms of flexural strength and elastic modulus. This sample even shows higher strength than some well-known commercial sealants such as Filtek Supreme XT Flow (FS = 115 MPa) and Clinpro (FS = 50 MPa) [63]. The high inherent strength of YSZ nanoparticles and their good distribution within the polymer matrix are the main factors contributing to the enhanced strength of the YSZ-containing sealants [64]. Addition of ε-PL to sealants largely reduces the flexural strength and elastic modulus suggesting the adverse effect of ε-PL on the polymerization process, which causes the weakened strength. However, this effect does not seem to be significant ($p > .05$) on the compressive strength of the sealants. The elastic modulus and flexural strength of the sealants decline upon incorporation of SrF_2 nanoparticles as well. This is in agreement with the results reported by Xu et al. [65,66]. They concluded that the addition of CaF_2 nanoparticles (0 to 40%) to resin-dental composites resulted in a continuous reduction of flexural strength from about 150 to 90 MPa. The compressive strength of the sealants shows an upward trend due to loading both SrF_2 and YSZ nanoparticles (up to 10 wt%). By increasing the nanoparticulate filler content from 10 to 20 wt%, the compressive strength falls down. Due to the high surface activity of nanoparticles, the agglomeration probability goes up when the nanoparticle content increases. Consequently, the sealant cannot withstand the high applied forces because of the lack of uniform force distribution resulted from the non-uniform distribution of load-bearing nanoparticles within the polymer matrix [32]. Andreotti et al. [67] concluded that although nanoparticles could reinforce the polymer-based composites, an appropriate level of the nanoscale filler must be incorporated due to the high surface energy and chemical reactivity of the nanoparticles. By addition of excess nano-filler to the polymer matrix, nanoparticles may agglomerate and the agglomerated particles act as stress-concentrating centers in the resin matrix, thereby decreasing its mechanical strength. Supporting data may also be found in other studies, where Xia et al. [32] and Sodagar et al. [68] showed that agglomerations of nanoscale particles, however, often attain micrometer scales limiting improvements to the mechanical properties of resin composites.

As light curable polymer-based restorative materials which are applied intra-orally, all formulated sealants with a maximum filler content of 10 wt% comply with the minimum requirements of ISO-4049 (flexural strength ≥ 50 MPa).

A dental sealant of high quality should possess essential properties resembling the structural, physical, and mechanical characteristics of dentin and enamel. Xu et al. [69] measured the elastic modulus of enamel to be 94 GPa. They found that the elastic modulus is significantly related to the orientation of dental microstructures. The elastic modulus calculated for all formulated sealants in this study is far less than the reported elastic modulus of enamel. This seems to be of less importance for pit-and-fissure sealants, as it has been suggested that dental sealants do not suffer from severe stress [70].

It can be argued that the most important limitation in the use of resin-based restorative dental materials in all of their types, especially resin composites and pit-and-fissure used for posterior teeth restoration, is their shrinkage during light-curing, which results in poor sealing and subsequently secondary caries occurrence. The polymerization shrinkage takes place because Van der Waals bonds are converted to covalent bonds during the polymerization of monomers and the resulted volume changes cause the material contraction [71]. Fig. 2 shows the percentage of polymerization shrinkage measured for the formulated sealants. As can be seen, by increasing the amount of the loaded nanoparticles, the polymerization shrinkage reduces, which can be due to a reduction in the weight percentage of monomers and subsequently enhanced polymerization. The percentage of shrinkage rises with addition of ε-PL to the sealants, but the recorded change seems to be not significant ($p > .05$) in most of the formulated materials. Therefore, it can be concluded that the adverse effects and possible disturbances of the polymerization process due to the incorporation of ε-PL are not sufficiently significant to cause a dramatic fall of the key parameters of the sealants. The formulated sealants exhibit relatively similar shrinkage percentages and in some cases fewer shrinkages than those of commercial sealants such as Tetric Flow (PS = 3.98–4.39%) and UltraSeal XT (PS = 5.07–5.63%) [72].

Water absorption and solubility are two basic parameters in the evaluation of restorative dental materials. Water absorption can have a significant effect on the dimensions of composite materials and cause a radial stress. Versluis et al. [73] showed that in polymer composite resin, the shrinkage due to polymerization could be compensated by an acceptable level through the expansion due to water absorption. So the material selection in terms of hydrophilicity and hydrophobicity will play a critical role in resin-based materials. As it has been suggested that dental resin composites should be formulated to have sufficient expansion (due to water absorption) to compensate the polymerization shrinkage, but the expansion should not exceed a certain limit to cause extra stress [74]. Fig. 3 shows the water absorption and the solubility measured for different composite sealants. Here, the water absorption declines by inclusion of nanoparticles, especially YSZ nanoparticles. This is in line with the results obtained by Bociong et al. [75] and Alshali et al. [76] who reported that the water sorption climbed in resin composites by increasing filler content. Water sorption is a diffusion-controlled phenomenon and multi-factorial process, which is mainly influenced by polymer matrix composition as well as the type, content, size, and shape of filler particles [77]. The most likely site of water accumulation is the filler-polymer interface. Water diffuses through the resin and reacts with the filler at the filler-polymer interface. Therefore, water uptake is much more affected by a poor filler-polymer interfacial adhesion as it should dramatically go down when matrix and filler particles are effectively coupled by a proper crosslinking agent [76,77].

Addition of SrF_2 nanoparticles to the sealants leads to increase of sealants' solubility pertaining to the susceptibility of the nanoparticles to release a vast number of strontium and fluoride ions, which subsequently leads to increased solubility [34]. Composite sealants containing 10 wt% YSZ nanoparticles show a relative increase in solubility compared to unfilled resins. ε-PL contributes to increased solubility and water absorption in the sealants owing to its hydrophilic nature and high solubility. Solubility is mainly due to the elution of residual monomers, initiators, and elements from filler particles. Solubility and water sorption are expected to change in the same way, since the

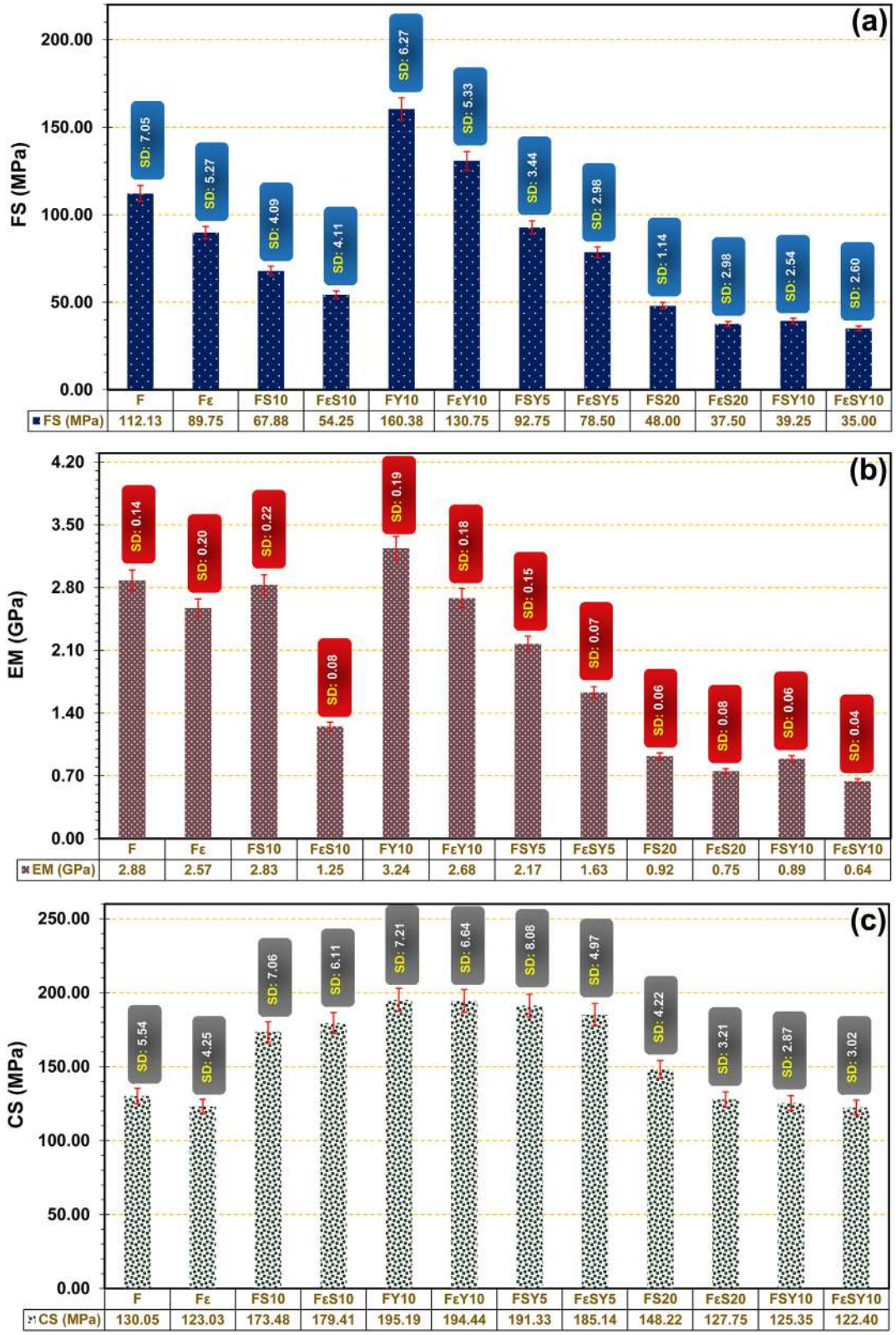

Fig. 1. (a) Flexural strength, (b) elastic modulus, and (c) compressive strength of the formulated sealants.

solvent penetrates the resin for leachable components to be able to release [75,76]. However, here we observe a negative correlation between solubility and water sorption, which is in line with observations made by Boaro et al. [78] and Papageorgiou et al. [79]. Overall, the reported values for water absorption and solubility of all formulated sealants are quite below the maximum values specified in ISO-4049 for water absorption ($\geq 40\ \mu g.mm^{-2}$) and solubility ($\geq 7.5\ \mu g.mm^{-2}$) of resin-based restorative materials.

Microstructure and surface integrity have significant effects on the properties of resin-based composites. Naturally, composites with integrated microstructure and free of defects (porosity, microcracks or gaps) exhibit more suitable physical-mechanical characteristics. Due to

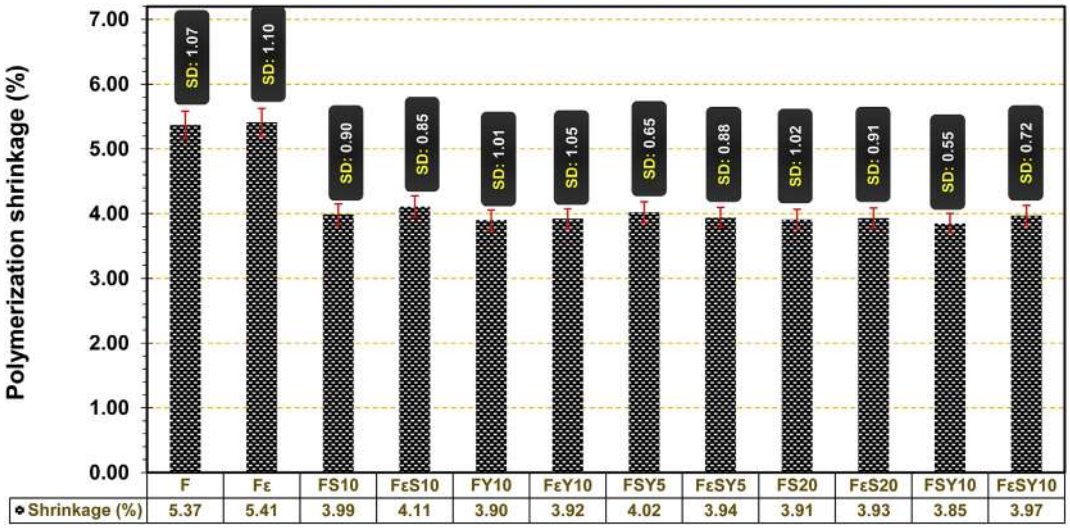

Fig. 2. Polymerization shrinkage of the formulated sealants.

the high surface area and significant surface activity, nanoparticles, especially after drying, show a high tendency for agglomeration and aggregation [80]. The distribution quality of nanoparticles within polymer matrixes depends largely on their separation during the mixing process [81]. Although in this study, a smooth and uniform paste with a good viscosity was obtained after mixing the nanoparticles with monomers, however, we were trying to distribute a hydrophilized substance (through surface modification and silanization) in a

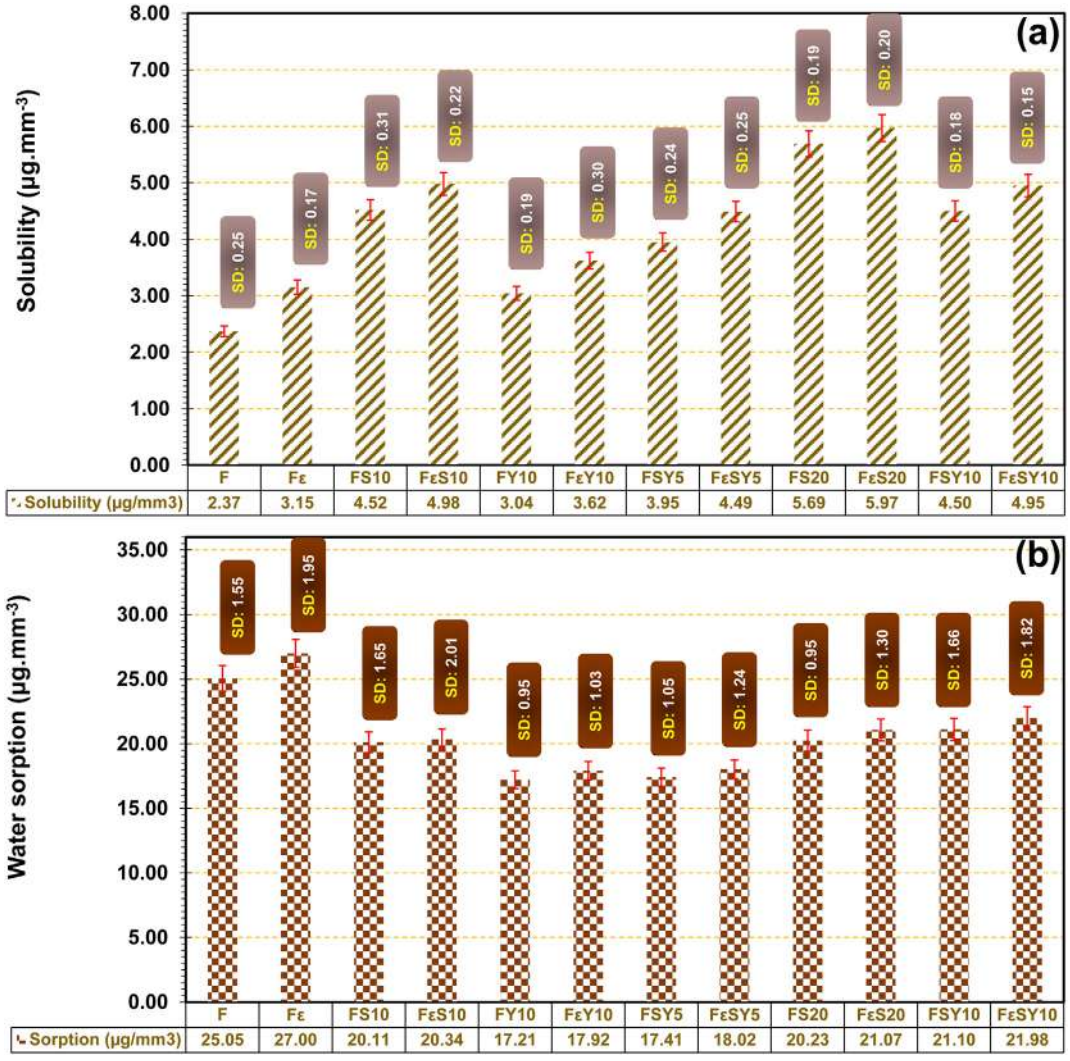

Fig. 3. (a) The solubility and (b) water sorption measured for nanocomposite sealants.

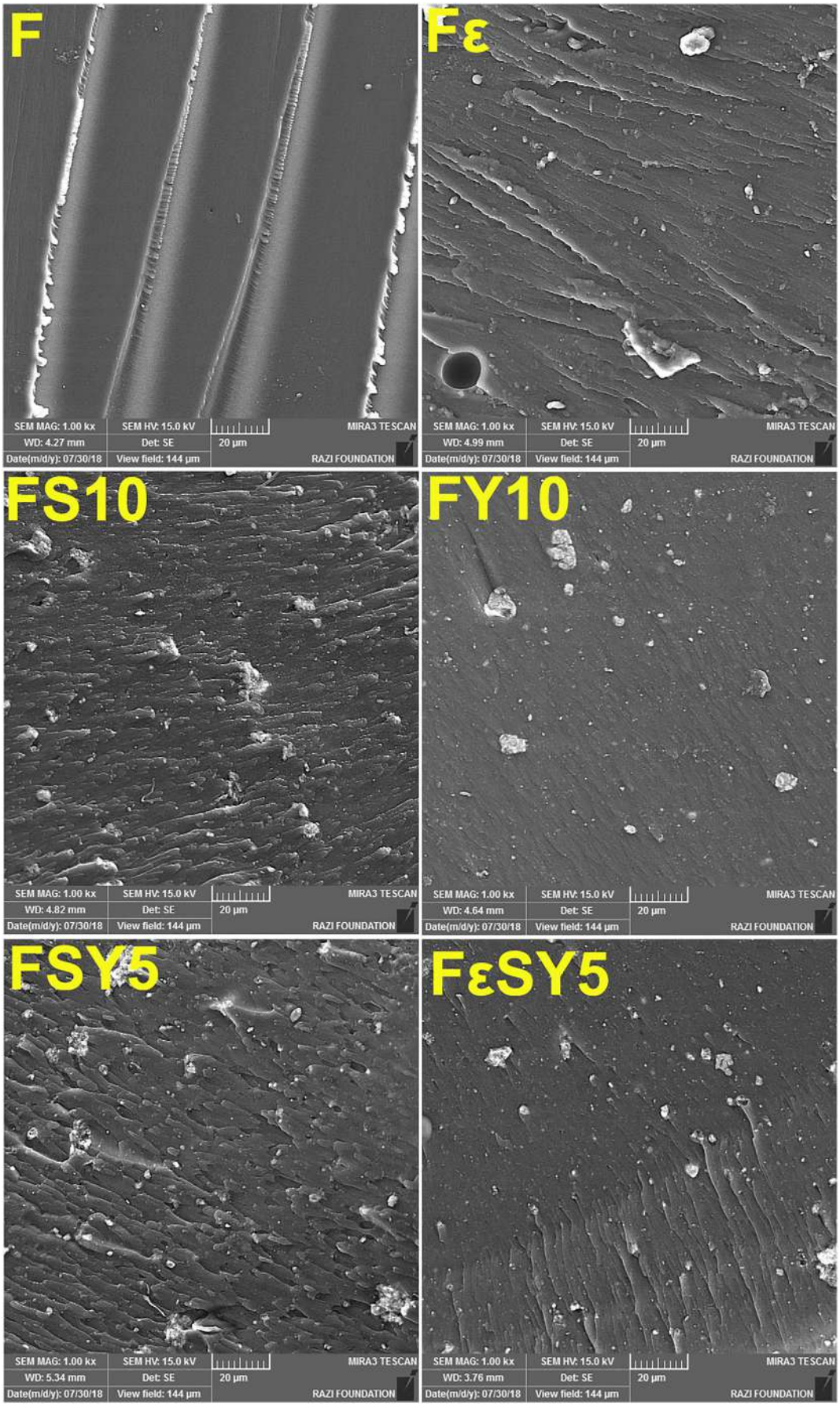

Fig. 4. FESEM micrographs of the fracture surface of the formulated nanocomposite sealants.

hydrophobic bed (polymer), and this is a challenge in view of materials chemistry! As a result, the presence of agglomerated nanoparticles within polymer seems to be unavoidable despite the surface modification of nanoparticles beneficial to uniform distribution of nanoparticles in the polymeric matrix [81,82]. FESEM micrographs in Fig. 4 obtained from the fracture surface of the nanocomposite sealants provide visual confirmation of the recent explanations. As can be seen, the unfilled resin (F) exhibits a smooth, uniform surface almost free of the hole, roughness, and porosity. There are some particulate regions in the unfilled resin containing ε-PL (Fε) that are related to the accumulated polypeptide particles. Upon incorporation of the nanoparticles, whether SrF_2 or YSZ, the surface roughness gradually increases and in spite of the proper distribution of the nanoparticles in the polymeric, nano-agglomerates can be seen yet. These are the weak points of the composite sealants which particularly impose their effects on the flexural strength. All formulated sealants are almost free from microcracks, gaps, cavities, and porosity. Also, there cannot be observed any crack or gap at polymer-nanoparticle interfaces suggesting the proper chemical bonding of the components affected by the proper surface modification of the nanoparticles.

In order to further investigate the distribution of nano-SrF_2, nano-YSZ and -PLε nanoparticles within the polymer matrix, a 2D elemental distribution map for FεSY5 sealant is presented in Fig. 5. Here, carbon (C), zirconium (Zr), strontium (Sr) and nitrogen (N) elements are representative of the polymer matrix, SrF_2 the nanoparticles, YSZ nanoparticles, and ε-PL (although nitrogen is also present in UDMA monomer). Two-dimensional maps clearly illustrate the uniform distribution of components proportional to their weight percent within the polymer matrix. It is worth to mention that the sample FεSY5 contains SrF_2 and ZrO_2, besides the presence of the resin matrix and polylysine additive. Thus, it was selected to analyze by EDS image map technique to show the distribution of all constituents.

3.2. Antibacterial activity of the sealants

Today's advanced dentistry demands durable dental materials because they reduce the patient's pain and cost, and the number of visits to the dentists. Both restorative materials themselves and bacteria are considered as the main factors of failure of tooth restoration. Nowadays, secondary caries are known as the major factor of the failure of restorations made with resin composites and glass ionomers [82]. Occurring at the interface of restorative materials and tooth cavity, secondary caries are the direct consequence of demineralization of the tooth structure due to the invasion of plaque bacteria (acid producing bacteria), especially *Streptococcus mutans*, in the presence of fermentable carbohydrates. Likewise, *Enterococcus faecalis* retains in root canals of the carious tooth and elicits continuous asymptomatic infections paving ways to endodontitis. Therefore, the key to durable restorative materials could be in the hands of antibacterial additives like fluoride salts and quaternary ammonium salts [83,84]. In the present study, we have tried to induce a strong antibacterial activity in the formulated pit-and-fissure sealants using a new approach based on SrF_2 nanoparticles–ε-PL combinations. As previously described in Section 2.9, for a comprehensive evaluation of antibacterial activity, two kinds of bacteria including Streptococcus mutans and Enterococcus faecali were examined. The ability of mutans virulence factors to synthesize adhesive glucans and create acids lead to the demineralisation of dental tissues, subsequently, starting dental caries [85]. In addition, *E. faecalis* as a facultative anaerobe, is one of the resistant bacteria commonly found in the root canals of teeth with endodontic treatment failure [86]. *E. faecalis* is resistant to a broad range of antibiotics [87]. Moreover, it has been found that the preservation of optimal cytoplasmic pH levels of this kind of bacteria (because of the effective proton pump mechanism) may take part in their resistance to the antimicrobial effects of calcium hydroxide [88].

The difference in “N” values against the two tested types of bacteria can be attributed to the virulence of each species and to the differences in the chemical composition and structure of the bacteria cell walls that results in distinct bacterial sensitivities toward fluoride and ε-Poly-L-Lysine (ε-PL) (Fig. 6). Lower “N” values were achieved against *E. faecalis*. The calculated viable bacteria for each composite show that, for FεSY5, “N” value is significantly higher than those of the other specimens. The number of colonies of all tested bacteria is reduced by the addition of ε-PL and SrF_2 nanoparticles into the formulation of the composites. “N” ranged between 1.6 and 5.1 following the order *E. faecalis* < S. mutans.

ε-PL is a homopolymer of L-lysine with a polymerization degree ranging from 25 to 35 that its structural units (lysine) are interconnected through ε-amino and α-carboxyl groups. ε-PL is completely biodegradable, non-toxic, edible, water-soluble, and thermally stable. ε-PL is a powerful antibacterial agent and is widely used in the food packaging industry due to its broad-spectrum antibacterial activity against a variety of Gram-positive and Gram-negative bacteria, yeasts and fungi [89]. Today, it is well-known that the anti-bacterial activity of the ε-PL is closely related to the length of the lysine chains, and at least 10 repeating units of lysine are critical for inducing antibacterial properties. The mechanism behind the antibacterial activity of the ε-PL is not fully understood, but the scientific literature suggests that the absorption of ε-PL on the outer wall of the cells plays an important role in its antibacteriality. Recent studies revealed that ε-PL, as a cationic polypeptide, interacts and penetrates the cell through ionic absorption on the surface of negatively-charged cells, followed by reactions that involve the degradation of the lipopolysaccharide (LPS) layer and subsequently cell membrane permeability and abnormal distribution of cytoplasm [89,90]. This mechanism applies more to Gram-negative bacteria. Studies also emphasize the very favorable antibacteriality of ε-PL against the Gram-positive bacteria but without any change in the cell wall. Although the exact mechanism is still unclear, it seems that the antibacterial activity of the ε-PL against Gram-positive bacteria is more likely to be associated with a disturbance in metabolism and protein synthesis in bacteria [91].

On the other hand, fluoride ion can affect bacterial metabolism in several ways with different mechanisms. Based on a possible mechanism, fluoride can act directly as an inhibitor for enzymes such as enolase or links to peroxidases. Peroxidases of many oral bacteria are insensitive to fluoride. Therefore, relying on another likely mechanism, fluoride can form complexes such as AlF_4^- which is known as the most important inhibitor of F-ATPases. ATPases resemble the phosphate group in ATP and form ADP complexes in active and hydrophilic sites of enzymes. ADP complexes inhibit the metabolism of bacteria and subsequently cause cell death. This kind of fluoride inhibitory effect has been observed in bacteria such as *Streptococcus mutans* and *Lactobacillus casei*. However, the function of fluoride, which is well-known for its anti-caries properties, mostly comes from its weak acidity. Fluoride increases the permeability of the bacterial membrane to the protons and, by interfering with the activity of F-ATPase in the production of protons, leads to the acidification of the cytoplasm and acidic inhibition of the enolases. In fact, fluoride drastically reduces the endurance of bacteria under acidic conditions of plaque and prevents glycolysis of bacteria, which eventually results in metabolism stoppage and bacterial death [34,92].

In this study, it appears that the fluoride released from the formulated sealants act as a pioneer and open the way for the ε-PL molecules to more easily attach to bacteria and induce their inhibitory effect through enhanced cell permeability and permeability provided by fluoride [92]. This exemplifies a positive collaboration or synergy between fluoride ions and ε-PL molecules toward enhanced antibacteriality, which is referred to as *the multiple hurdle approach* in the literature [93]. In fact, according to the results (Fig. 6), the synergic effect of ε-PL and fluoride in the composition of Bis-GMA–-TEGDMA–UDMA mixed monomers even represents a better N value compared to the other resin-based composites with different

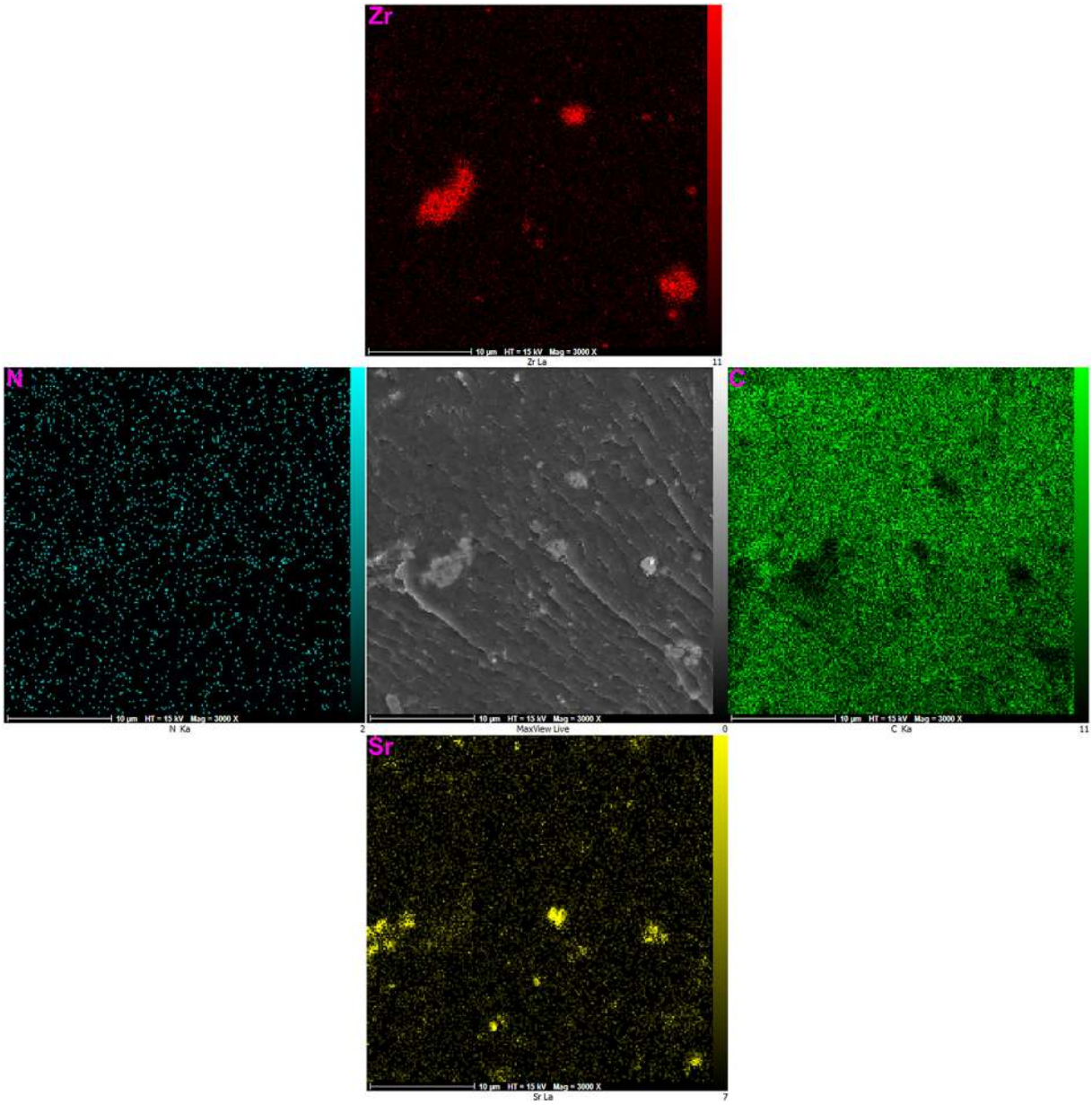

Fig. 5. 2D elemental map of FεSY5 nanocomposite sealant.

composition which have been by other researchers [94–96].

3.3. Fluoride release study

In recent years, dental restorative materials with the capability of fluoride release have been considered by many researchers and dentists. Today we are well aware that fluoride-releasing materials exhibit a high potential to combat tooth decay. Fluoride substitutes for hydroxyl groups in hydroxyapatite as the main ingredient of enamel and dentin. The result of the substitution is fluorapatite, which has a much lower solubility and more acidic resistance than hydroxyapatite leading to increased resistance of tooth against the challenging oral conditions. In addition, as mentioned earlier, fluoride counteracts biofilm-producing bacteria and, through the disturbance in the plaque production process. It provides better conditions for tooth remineralization [12]. Studies have revealed that glass ionomer cements and resin-modified cements show a higher and more stable fluoride release than composite resins. Therefore, in recent years, in particular, efforts have been made to strengthen the release of fluoride from composite resins, as they show more suitable physical-mechanical properties than glass-ionomer cements [12,97].

Fig. 7(a) shows the fluoride release patterns from FSY5 and FεSY5 nanocomposite sealants in two different media, namely deionized water (pH = 6.9) and acetic acid (pH = 4.0) over 98 days. As expected, the highest ion release occurs in the early days, and then gradually decreases until it reaches a steady state. The maximum release in the aqueous medium occurs on the third day and on the first day in the acidic environment, which could be due to faster dissolution of SrF_2 nanoparticles in acidic media [69]. Adding ε-PL to FSY5 nanocomposites does not affect the fluoride release adversely. The data even shows the enhanced cumulative fluoride release rate from FεSY5 in the acidic environment after 98 days.

The cumulative release profiles of fluoride from FSY5 and FεSY5 nanocomposites are presented in Fig. 7(b). The average amount of released fluoride for FSY5 and FεSY5 nanocomposites is calculated to be 0.89 and 0.86 $\mu g \cdot cm^{-2} \cdot day^{-1}$ in the aqueous medium, and 1.49 and 1.58 $\mu g \cdot cm^{-2} \cdot day^{-1}$ in acidic medium, respectively. Comparing the obtained values with the reported amounts for CaF_2-containing

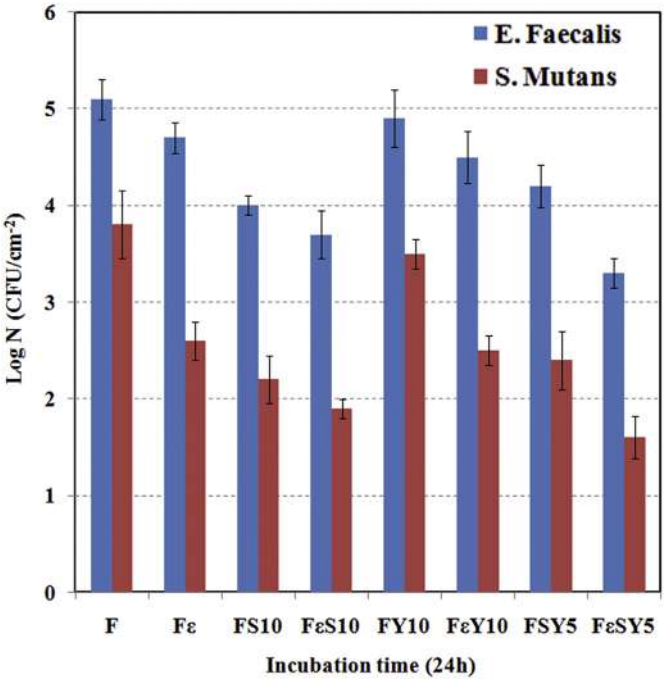

Fig. 6. The log CFU/cm^2 of tested *S. mutans* and *E. faecalis* bacteria on the experimental composites after 24 h incubation.

nanocomposites (0.50–1.13 μg·cm^{-2}·day^{-1}) by Xu et al. [66], reveals the reinforced release of fluoride from the formulated nano-sealants in this study.

3.4. Biocompatibility of the sealants

The behavior of hMSCs in the presence of FSY5 and FεSY5 extracts was investigated by MTT cytotoxicity assay and acridine orange staining. The optical density (OD) of the cells cultivated in the presence of the sealant extract is shown in Fig. 8(a). Based on the results, a significant difference ($p < .05$) was observed between OD values in the absence and presence of the sealant extracts. The reported OD values after 7 days are equivalent to the cell viability percentages of 72.9 and 80.3% indicating moderate to low toxicity of FSY5 and FεSY5 sealants. Several studies have confirmed the cytotoxicity of resin-based dental materials, which has been mainly attributed to the chemicals leached from materials, in particular, free monomers such as bisphenol A and TEGDMA [98]. Al-Hiyasat et al. [99] showed that both Feltik Flow and Tetric Flow commercial sealants were severely cytotoxic (based on MTT assay) and they were both more cytotoxic than their traditional composites. Attik et al. [98] revealed that cytocompatibility of resin composites toward primary human gingival fibroblasts was directly related to the amount of resin present in composites and it was mainly attributed to the release of residual TEGDMA rather than the BPA derivatives. By comparing OD values obtained for FSY5 and FεSY5 extracts, it can be deduced that cellular life rises upon incorporation of ε-PL, which pertains to the proven role of polylysine in the enhancement of cell adhesion and growth [100]. Fig. 8(b-d) shows the fluorescence micrographs of the stained hMSCs. The micrographs related to the cells cultured in the sealant extracts show some red spots indicating dead cells may be due to trace toxic products released from sealants. In spite of this, no change is observed in the cell morphology and the mesenchymal stem cells retain their fusiform morphology. In comparison, the formulated sealants show lower cytotoxicity than reported cases. This can be partly because of that MSCs withstand more the harmful chemicals released from dental resin composites as Roman et al. [101] concluded that oral mesenchymal stem cell lines grew well on the surfaces of both commercial and experimental resin composites with no signs of cytoskeleton disruption.

4. Conclusion

Nanocomposite sealants were formulated using Bis-GMA, TEGDMA and UDMA monomers and camphorquinone photoinitiator, as well as synthetic SrF_2 nanoparticles, YSZ nanoparticles, and ε-PL. The antibacterial activity of the nano-sealants against *Streptococcus mutans* was increased by adding SrF_2 and ε-PL, but none of the formulations reached the reasonable value except in formulations containing both of

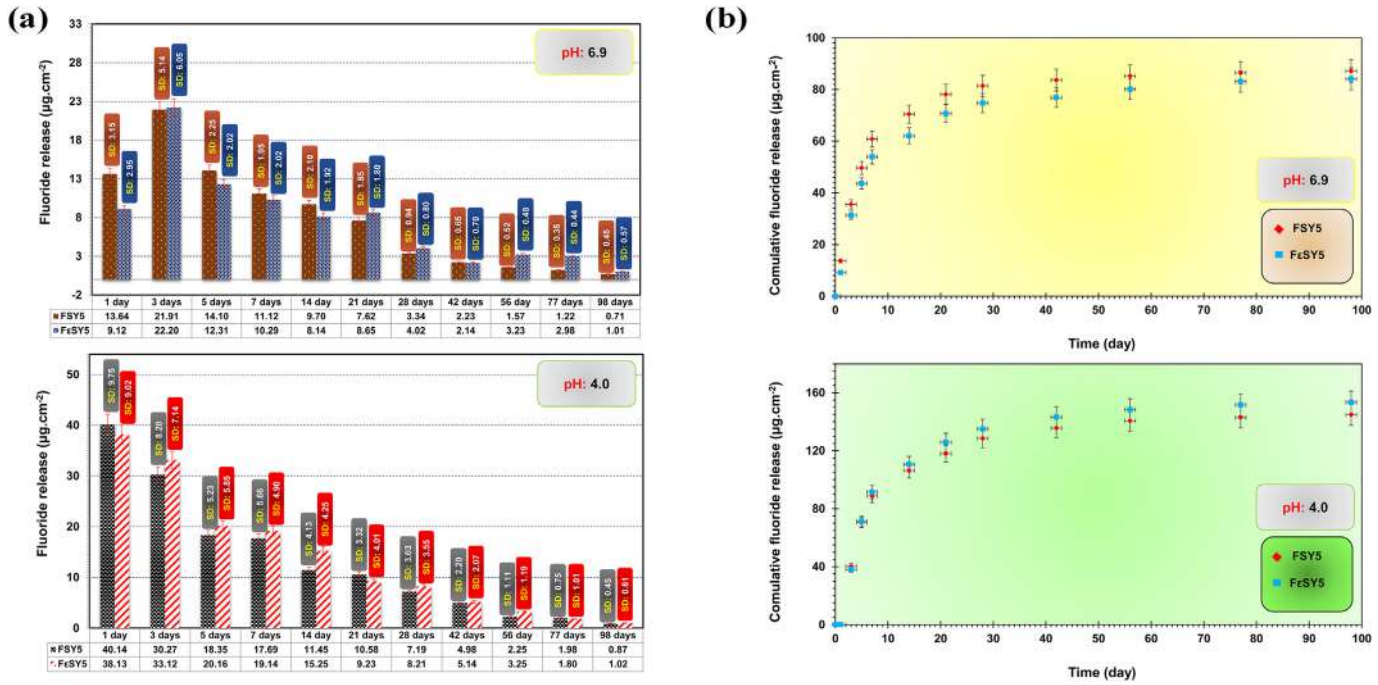

Fig. 7. (a) The amount of fluoride ion released from FSY5 and FεSY5 sealants in aqueous (pH 6.9) and acidic (pH 4.0) media over 98 days. (b) Cumulative release of fluoride from FSY5 and FεSY5 sealants in aqueous and acidic media over 98 days.

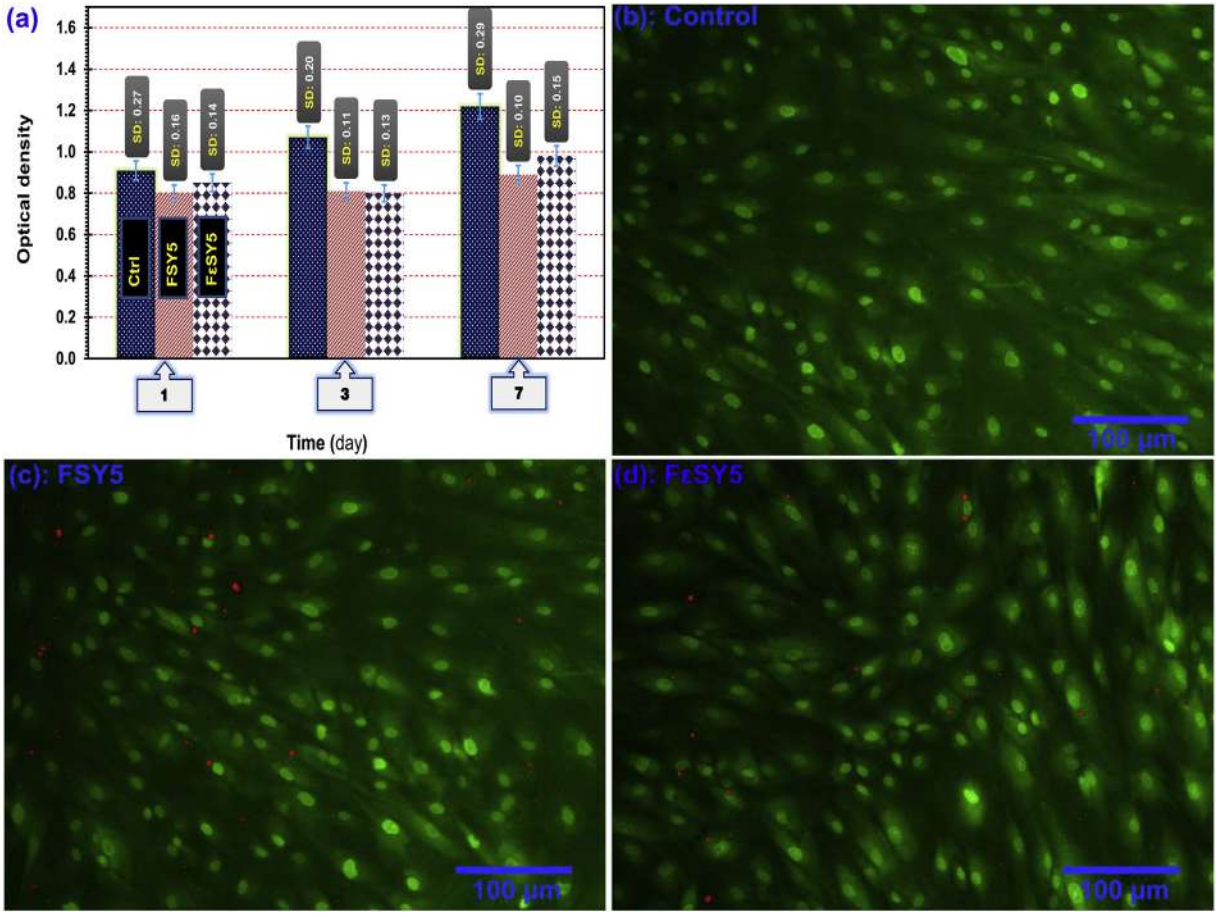

Fig. 8. (a) Optical density of hMSCs, (b), (c), and (d) fluorescence micrographs of acridine orange-stained hMSCs after culture in the absence (control) and presence of FSY5 and FεSY5 extracts.

additives. These observations revealed a synergistic effect between SrF_2 and ε-PL. The inclusion of SrF_2 and ε-PL was accompanied with mechanically weakening of the sealants that was partly compensated by incorporation of YSZ nanoparticles (up to 10 wt%). It can be claimed that our hypothesis is verified as a whole. Based on physicomechanical and antibacterial properties, the sealant composed of 5 wt% SrF_2, 5 wt % YSZ and 0.5 wt% ε-PL was selected as the optimal sealant and was evaluated for fluoride release and cytotoxicity. Owing to the incorporation of synthetic SrF_2 nanoparticles with high specific surface area, the sealants showed an enhanced fluoride release rate as compared to that reported for composites containing CaF_2 nanoparticles and some dental cements. The cytotoxicity test showed moderate cytotoxicity of about 30% for optimal nano-sealants, which declined to ~20% due to addition of ε-PL.

CRediT authorship contribution statement

Saeed Hesaraki: Supervision, Writing review & Editing. **Mohammad Karimi:** Writing - original draft, Software, Methodology. **Nader Nezafati:** Writing - review & editing.

Declaration of competing interest

The authors state that there is no conflict of interest for this manuscript.

Acknowledgment

The support of Iran National Science Foundation under Grant No. 95838568 is gratefully acknowledged.

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