

Article

Modulus, Strength and Cytotoxicity of PMMA-Silica Nanocomposites

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Received: 12 May 2020; Accepted: 15 June 2020; Published: 23 June 2020



Abstract: Key advantages of Poly(methyl methacrylate)—PMMA for denture application are related to aesthetics and biocompatibility, while its main deficiency is related to mechanical properties. To address this issue, SiO₂ nanoparticle reinforcement was proposed, containing 0 to 5% nanosilica, to form nanocomposite materials. Flexural strengths and elastic moduli were determined and correlated to nominal nanoparticle content and zeta potential of the liquid phase nanoparticle solutions. Another issue is the biocompatibility, which was determined in terms of cytotoxicity, using L929 and MRC5 cell lines. The addition of nanoparticle was proved to be beneficial for increasing flexural strength and modulus, causing a significant increase in both strength and moduli. On the other hand, the formation of agglomerates was noted, particularly at higher nanoparticle loadings, affecting mechanical properties. The addition of nanosilica had an adverse effect on the cytotoxicity, increasing it above the level present in unmodified specimens. Cytotoxic potential was on the acceptable level for specimens with up to 2% nanosilica. Consequently, nanosilica proved to be an effective and biocompatible means of increasing the resistance of dental materials.

Keywords: nanocomposite; mechanical properties; cytotoxicity; nanosilica

1. Introduction

Poly(methyl methacrylate) (PMMA) is the most frequently used material for dentures today. Advantages of PMMA over previously used materials [1] include its aesthetics and biocompatibility. However, PMMA mechanical properties, particularly the strength and ductility are not optimal, leaving wide opportunities for further improvements. Indeed, the conducted studies found that over a half of them fail in the first three years [2]. Having in mind that dentures are mainly used by elderly people, often having limitations in mobility, the repair of fractured dentures presents an inconvenience and a significant problem. Therefore, increasing mechanical properties of denture material is of great significance, without compromising aesthetics and biocompatibility [3].

There are a number of ways to improve mechanical properties of PMMA materials, some being technological aftertreatments, while some used additional materials to improve properties of PMMA, creating composites or nanocomposites. A very attractive way of improving PMMA mechanical response is the application of microwave aftertreatment and water bath post-polymerization. These aftertreatments were the topic studied in the works by Vergani et al. [4], Balos et al. [5,6] and Urban et al. [7]. In these studies, it was found that PMMA mechanical properties can be increased by treatments after the curing was finished. Heating, by means of hot water bath or microwave

irradiation proved to be effective, particularly for increasing mechanical properties of autopolymerized PMMA with a higher amount of unconverted monomer. By applying these technologies influence the marked reduction in residual monomer. Namely, the most significant reason why PMMA with residual monomer does not have convenient mechanical properties is that residual monomer is an unconverted polymer material. This acts as empty space, with the effect of a microvoid. As such, under load, it acts as a stress concentrator, actually weakening the material [8,9]. Another issue is the gum irritation and possibly even an allergic reaction to the pockets of monomer present in the PMMA [10].

Another opportunity for hot-cured PMMA, containing initially a lower amount of unconverted monomer mechanical properties can be increased by fibers or nanoparticle addition, effectively turning PMMA into a PMMA—based composite or a nanocomposite material. Carlos and Harrison [11] introduced ultra-high molecular weight polyethylene (UHMWPE) fibers into PMMA acrylic resin denture base material. However, some mechanical properties, such as hardness and impact strength were reduced compared to the PMMA matrix. Furthermore, the introduction of hydroxyapatite in studies by Chow et al. [12,13] of up to 5 wt.% can have variable effect on mechanical properties. It was shown that it can influence the increase in fracture toughness, however, flexural strength decreased.

The most likely explanation for such behavior is the formation of agglomerates that formed at these relatively high nanoparticle loadings. Other types of particles were tested, such as ZrO_2 and Al_2O_3 in the studies of Ayad et al. [14], Ellakwa et al. [15] and Alhareb and Ahmad [16]. Ayad et al. [14] reported that the relatively high loading of 15% ZrO_2 nano particles can influence the significant improvement in impact strength of PMMA. On the other hand, Ellakwa et al. [15] successfully applied Al_2O_3 nanoparticles in order to statistically significantly increase the flexural strength of PMMA. Finally, an interesting approach was applied by Alhareb and Ahmad [16], who reinforced PMMA with various ratios of $\text{Al}_2\text{O}_3/\text{ZrO}_2$ nanoparticles. It was shown that nanoparticles have a beneficial effect on increasing the flexural modulus, strength and fracture toughness. On the other hand, tensile modulus and strength were reduced. Combining different types of nanoparticles may be beneficial for reducing the tendency to form agglomerates and therefore be more effective in increasing mechanical properties. This is in accordance with the research by Efimov et al. [17].

One of the most widely used nanoparticle type in scientific community is nanosilica (nano- SiO_2). Several studies addressed the addition of nanosilica particles to PMMA resins. Yang et al. [18] and Balos et al. [19,20] found that nanosilica addition can have a significant influence on flexural strength, modulus, microhardness and fracture toughness. Relatively low nanoparticle loadings of up to 2% proved to be highly effective due to the formation of agglomerates at higher loadings, causing a non-homogenous distribution of nanoparticles, along with their polymer chain immobilization effect through the material section. Furthermore, relatively weak Van der Waals forces between nanoparticles in agglomerates cause the agglomerates to fracture under load, causing unstable crack propagation in the material, weakening it [21]. Nanoparticle addition may affect the materials biocompatibility, that is, the level of cytotoxicity. Namely, the addition of nanoparticles inevitably modifies the material structure, potentially affecting the nanocomposites biocompatibility. In the work by Yang and Nelson [22], PMMA/nanosilica composites were tested in tensile strengths and nanoparticle addition was proved to be beneficial in increasing the tensile strengths of over 50% and nearly doubling the modulus, while maintaining elongation at break.

In this study, PMMA denture relines resins were modified with nanosilica treated with hexamethyldisilazane (HMDS) providing hydrophobic properties. The selected increments of nanosilica content between individual specimens were relatively narrow, resulting in 12 nanosilica concentration overall, aimed at determining cytotoxicity threshold, a novelty by itself, aimed at detecting the influence of both SiO_2 and HMDS effect. Cytotoxicity tests were done on both mouse and human lung fibroblasts, with aim to find the more sensitive fibroblast type for these particular experimental conditions. Also, trends in flexural strengths and moduli values were found and correlated to the true particle size in the liquid phase. As such, this experimental setup was envisaged to give a comprehensive answer to the nanosilica effect on PMMA in both cytotoxic and mechanical response.

2. Materials and Methods

2.1. Materials

The base material used in this study is a two component PMMA, common in fabrication of acrylic dentures. This base material consisted of two components, the powder and the liquid. The powder had the composition consisting of pre-polymerized PMMA, pigments to emulate gums and benzoyl peroxide (chemical formula $C_{14}H_{10}O_4$). The liquid contained methyl-methacrylate (MMA) and 3,4-Ethylenedioxy-N-methylamphetamine (EDMA). When mixed together with the powder, radical polymerization occurs, forming the bulk material.

Modification agent was nanosilica (Evonik Aerosil R812S), having the nominal particle size of 7 nm. These particles were pre-modified with a surface layer of hexamethyldisilazane (HMDS) to provide hydrophobic properties and be able to be mixed with the liquid phase. The nominal surface area of nanoparticles was $220 \pm 25 \text{ m}^2/\text{g}$ [23]. These nanoparticles were added to the liquid component and subsequently, in a mixed form was mixed together with the powder. The amount of nanoparticles was added so that the content of nanoparticles in the prepared specimens was 0.02; 0.05; 0.1; 0.2; 0.5; 0.7; 1; 1.5; 2; 2.5; 3 and 5 weight % (wt.%). Also, 0% nanoparticles were used to fabricate control specimens, to assess the effect of the nanoparticle reinforcement. Nanoparticles were weighed by the Ohaus Adventurer Pro analytic balance, accurate to 0.0001 g, while the subsequent mixing was done in two stages: by the IKA C-MAG HS7 magnetic stirrer and the Vevor PS-20A ultrasonic bath. To find the true size of particles in the liquid component in the modified specimens, the Malvern Instruments Zetasizer Nano ZS device was applied.

2.2. Specimen Fabrication

The liquid component (unmodified and modified with nanoparticles) was then mixed with the powder with a spatula and left to rest for 10 min in a closed glass cup. When the dough-state of the mixture was reached, the material was poured in isolated flask halves at 40 °C, pre-filled with hardened plaster. The mold was made by boiling out 50 mm × 50 mm × 4 mm wax models. Subsequently, the flasks were closed and the whole set was pressed at the pressure of 80 bars and fixed by clamping.

Curing was done in boiling water for 45 min to reduce the residual monomer content. After cooling to room temperature in cold water, cutting was done by the Struers Discotom apparatus, the standard metallographic abrasive cutter, with subsequent finishing by grinding. Grinding was done on the Struers Knuth Rotor metallographic grinding device, with standard 1500 grit silicone carbide (SiC) abrasive papers. Final measurement to achieve 50 mm × 6.25 mm × 2.5 mm specimen size was done by the Hyundai Altraco micrometer accurate to 0.01 mm, to make the specimens comply with ISO-178 standard [24].

2.3. Characterization of Mechanical Properties

To determine the effect of the nanoparticle addition, flexural strength and modulus were determined. Flexural testing was conducted by means of a three-point bend testing procedure, by using the Toyoseiki AT-L-118B universal mechanical tensile testing machine. This device was equipped with a three-point bending tool. Crosshead speed was set at 1 mm/min, while flexural strength and modulus were calculated in accordance to Equations (1) and (2), commonly used in flexural testing of polymer, composite and nanocomposite specimens.

$$\sigma = 3F_{\max}L/(2BH^2) \quad (1)$$

$$E = FL^3/(4BH^3d) \quad (2)$$

where: σ is flexural strength of the material [MPa], F_{\max} is maximum load measured during flexural testing [N], L the distance between the flexural supports [mm], B and H are width and height of the samples [mm], E is the modulus of elasticity [GPa], while d is the deflection [mm] that corresponds to

the loading F. Flexural test was conducted in accordance to the common ISO-178 standard [24]. Overall, tests were conducted on the basis of 10 specimens, used for flexural modulus and subsequently flexural strength testing. In this paper, average values were reported, as well as corresponding standard deviations.

One-way analysis of variance (ANOVA) with Tukey's post-hoc test was done to assess the mechanical test results and find the mathematical significance between specimens modified with different nanoparticle loadings between each other and versus the control specimen. The significance level selected was set at the common $\alpha = 0.05$. Statistical analysis was done by applying the Minitab 19 software.

2.4. SEM and EDX Characterization

Fracture surfaces were examined by the JSM-6460LV scanning electron microscope (SEM) (JEOL, Tokyo, Japan), to give answers to possibly different fracture mode. Furthermore, even more important, it was aimed at finding crack propagation data. This would indicate the partial strength of the polymerized material versus pre-polymerized powder that form the tested nanocomposite material. Before the SEM analysis, the specimens were coated with gold using the SCD-005 device (Bal-Tec/Leica, Wetzlar, Germany). Energy-dispersive X-ray sepectroscopy (EDS) with INCA Microanalysis system was conducted to examine agglomerated nanoparticles and differentiate them from the polymer in the background.

2.5. Cytotoxicity Testing

Biocompatibility was determined by two classes of fibroblasts: L929 mouse fibroblasts (American Type Culture Collection CCL1) and MRC-5 human fibroblasts (American Type Culture Collection CCL 171). The cells were grown in DMEM (Dulbeccos modified Eagles medium), supplemented with 10% of FCS (fetal calf serum), antibiotic and antimycotic solution. Subculturing the cells was performed twice a week. The fabrication of a single-cell suspension was performed by using 0.1% trypsin in Ethylenediaminetetraacetic acid (EDTA). These cell lines were cultured in 25 cm² flasks, in a 100% humid environment containing 5% CO₂. The dye exclusion test (DET) with tarpan blue [25] was applied to determine cell number and the amount of viable cells. The viability of cells was above 90%. Material samples were extracted in 4 mL of DMEM Sigma (Dulbecco's modified eagle medium) without serum after 3, 5, 7, and 21 days. Duration of cell incubation was 48 h. Growth inhibition was determined in accordance with tetrazolium colorimetric MTT procedure proposed by Mosmann [26].

The viable cells were seeded into a microtiter with 96 wells at seeding density of 5103 cells per well, assuring logarithmic growth throughout the test. Serial dilutions of the various eluates in 100 µL volumes were added for 48 h at 37 °C and with the addition of 5% CO₂. After the treatment described in this procedure, 10 µL of MTT solution sterilized by filtration (5 mg/ml) was added to the wells. Incubation was done for 3 h at the temperature of 37 °C. Afterwards, suction removal of the medium and MTT was conducted. The formazan products were solubilised in 100 µL 0.04 M HCl in isopropanol. Subsequently, the plates were tested on a Labsystem Multiscan MCC340 spectrophotometer by using the wavelength of 540/690 nm, with the wells containing only the complete medium and MTT acting as blank. The testing was repeated twice. Inhibition of growth was calculated in accordance with Equation (3).

$$\%K = (A_{\text{test}}/A_{\text{control}}) \times 100 \quad (3)$$

where A is the absorbance of test and control sample, respectively.

3. Results and Discussion

3.1. Zeta Sizer Results

The results of zeta sizer of the modified liquid component are shown in Figure 1. In all charts, for all concentrations of nanoparticles, the smallest detected particles are considerably larger than the

nominal size of nanoparticles. That means that agglomeration occurs in all tested specimens, regardless of the nanoparticle content. These results suggest that the agglomeration occurs immediately after mixing of the nanoparticles and the liquid phase in spite of the presence of HMDS modification layer on the nanoparticles, magnetic stirring and ultrasonic bath treatment.

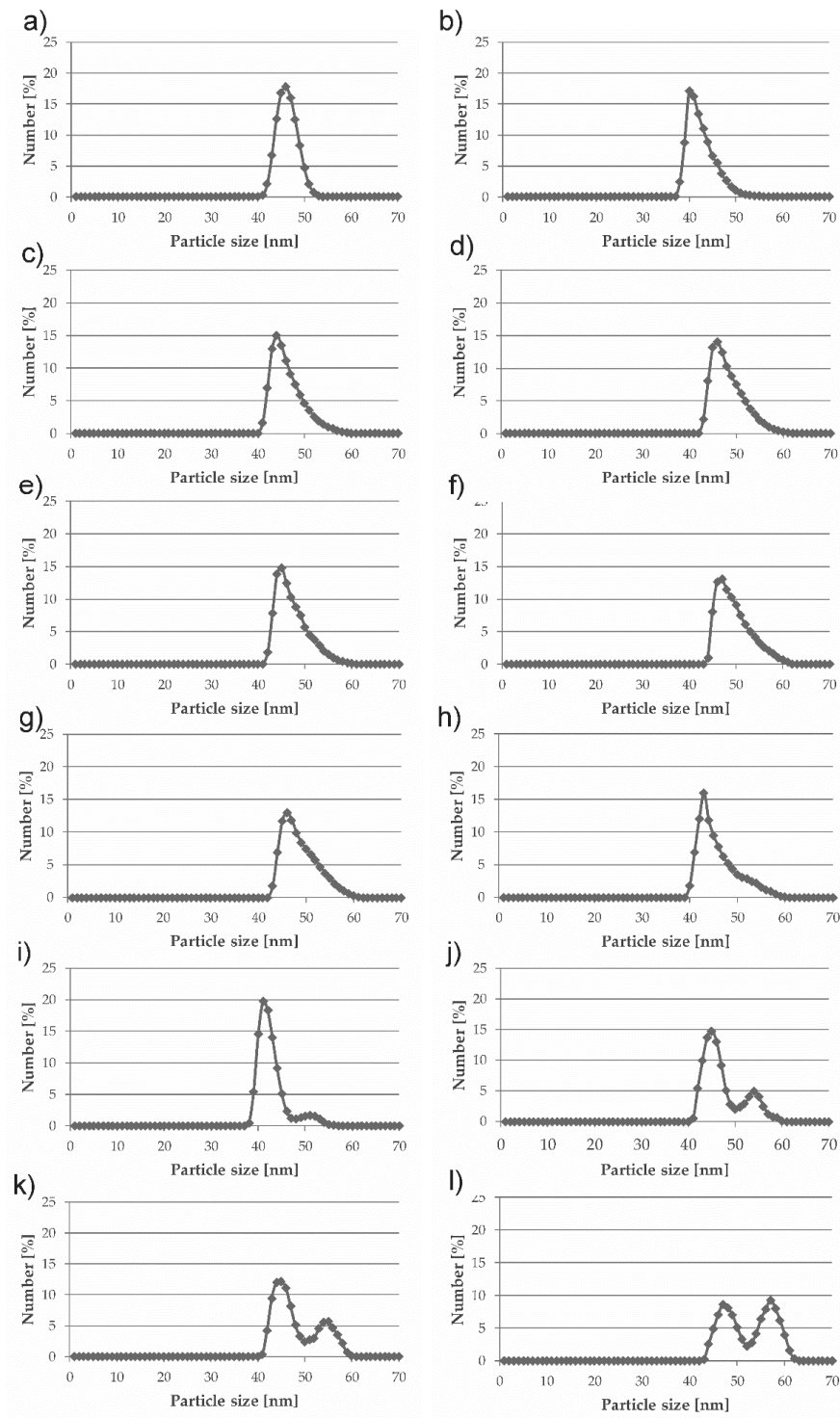


Figure 1. Zeta sizer results for modified liquid component that was subsequently mixed with powder, with a nanoparticle content of: (a) 0.02%; (b) 0.05%; (c) 0.1%; (d) 0.2%; (e) 0.5%; (f) 0.7%; (g) 1%; (h) 1.5%; (i) 2%; (j) 2.5%; (k) 3%; (l) 5%.

In Figure 1a–f, a single peak occurs with the maximum at 40–50 nm (specimens containing 0.02–0.7% nanoparticles). However, in Figure 1g,h, a transition occurs at the particle size of approximately 50 nm (1, 1.5%). At a higher nanoparticle content, a clear secondary peak occurs in the specimens modified with 2 and over 2% nanoparticles (Figure 1i–l). The secondary peak, which occurs beyond the particle size of 50 nm, becomes dominant in the specimen modified with 5% nanoparticles. This phenomenon suggests that an increase of nanoparticle content in the liquid phase increases the appearance of agglomeration as well, causing the occurrence of larger agglomerates in the nanocomposite material.

3.2. Mechanical Properties

The influence of nanoparticle content on flexural strength and modulus, along with the letter indication of statistical similarity between specimens is shown in Figure 2. Regarding statistical analysis, specimen groups that are marked with different letters are statistically different. The lowest mechanical properties were obtained in unmodified specimens which was expected. The highest flexural strength and modulus were obtained with 0.05% and 2% nanoparticles. The trends in flexural strength and modulus are similar, indicating that the effect of nanoparticle addition is similar in all modified specimens. Another observation in both charts depicting flexural strength and modulus is the notable waviness of average values: after the unmodified specimen 0, strength and modulus rises in specimen modified with 0.02% nanosilica, reaching maximum at 0.05%, after that the values drop in 0.1 and 0.2, rising again in 0.5%, dropping in 0.7 and 1%, rise again in 1.5 and 2%, and finally gradually drop down in 2.5, 3 and 5%.

The lowest values of flexural strength and modulus were obtained with the highest two nanoparticle contents of 3 and 5%. Also, it can be seen that standard deviations of tested specimens differ considerably. The smallest standard deviations were obtained in the unmodified specimen. As the nanoparticle content is increased, standard deviations are increased as well. This can be the result of somewhat stochastic effect of nanoreinforcement and possibly some degree of non-homogenous distribution. Similar findings were reported in [19,20,27], although [27] was aimed at increasing the properties of flowable dental composite by nanosilica addition.

The results of flexural strength and modulus are well correlated to each other and they follow a similar wavy pattern as the nanoparticle content is increased. This can be explained by zeta sizer results, which indicate that smaller particles in the liquid component also result in higher strengthening effect. Ideally, the particles detected would influence the appearance of a sharp peak, situated at the nanoparticle size as small as possible. Such distribution would indicate a relatively low agglomeration that would be effective in increasing the mechanical properties of the PMMA. The main strengthening effect of nanoparticles is reflected through the immobilization of polymer chains in the vicinity of nanoparticles.

Usually, around a micron distance around nanoparticle is considered as realistic for the reduction of polymer chain immobilization [28,29]. However, if the dispersion of nanoparticles is not optimal, that is, if relatively large agglomerates are formed, leaving areas of material without embedded nanoparticles and therefore without the mentioned polymer chain immobilization effect, the strengthening effect is not equally distributed throughout the material. This leaves considerable areas in the material microstructure unreinforced, without nanoparticles and without polymer chain immobilization. That means, there is a possibility for the crack to propagate between the reinforced fields, that is, through the unreinforced material, making the propagation easier.

After the specimen modified with 0.05% nanoparticles, the second specimen having the highest flexural strength and modulus is the specimen containing 2% nanoparticles, which is the second peak in the wavy trend of mechanical properties, Figure 2. This can be explained by the zeta sizer results, depicted in Figure 1. In Figure 1, the specimen modified with 2% of nanoparticles, a relatively sharp peak indicating around 20% of particles in the liquid phase has the size of 41 nm and there is also a relatively weak secondary peak at around 50 nm particle size, Figure 1i. This distribution is superior to both shown in Figure 1h (1.5% nanoparticles; 16% maximum at 44 nm) and Figure 1j

(2.5% nanoparticles; maximum at 45 nm and clear secondary peak at 54 nm). A more convenient particle distribution together with a higher nanoparticle content provides the specimen containing 2% nanoparticles with a higher flexural strength and modulus compared to the specimen containing 1.5% nanoparticles. A higher nanoparticle contents, 3 and 5%, provide lower mechanical properties due to a less convenient zeta potential shown in Figure 1k,l.

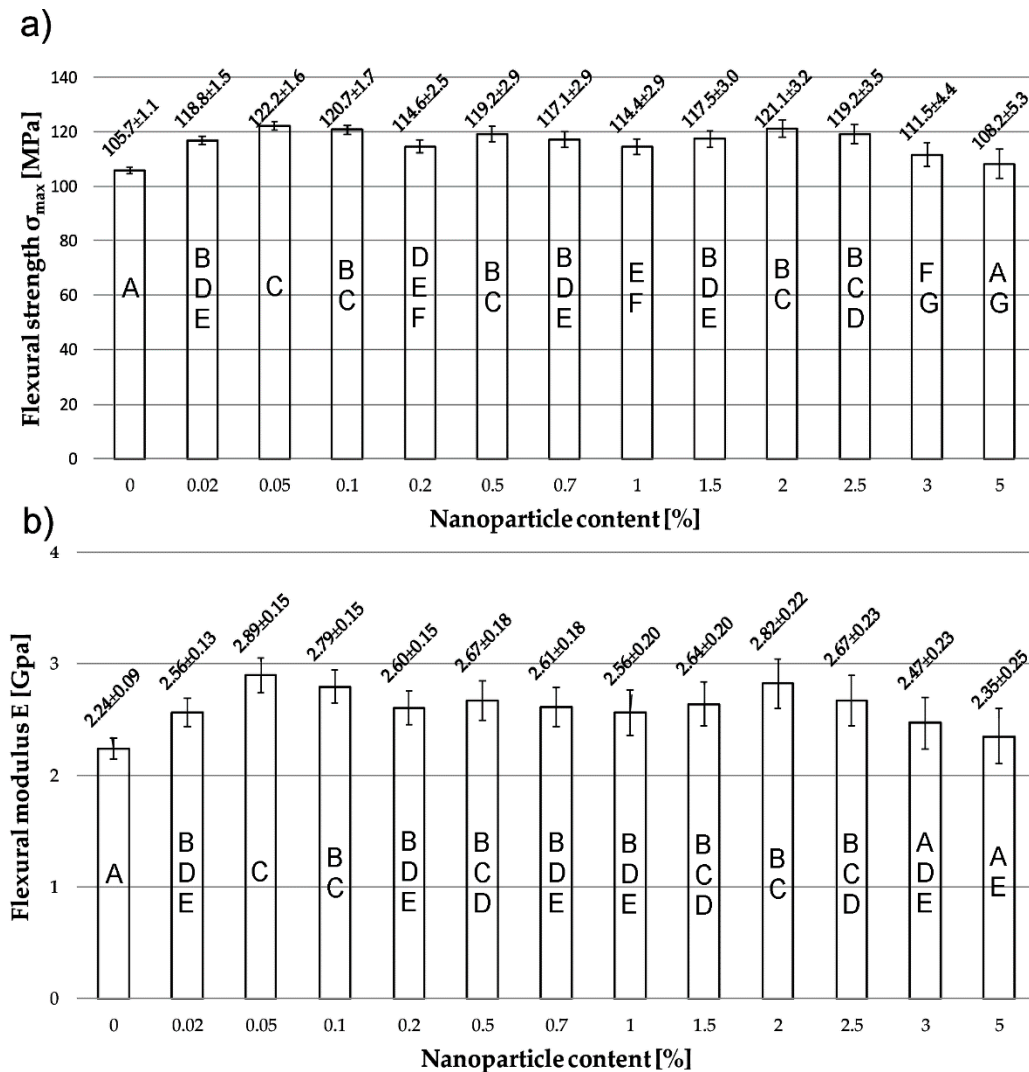


Figure 2. The nanoparticle content influence on flexural strength (a) and flexural modulus (b) of the unmodified and modified PMMA specimens. Letters indicate the statistical difference between specimens, whereby common letters indicate statistically insignificant differences.

3.3. Statistical Analysis

Statistical analysis results are shown in Figure 1, while in Tables 1 and 2, statistical analysis parameters are presented. These analyses confirmed that the majority of modified specimens exhibited statistically significant increase in flexural strength and modulus, mathematically showing the benefits from adding nanoparticles. The exception is the specimen modified with 5% nanosilica, which exhibits statistically similar flexural strength and modulus as the unmodified specimen. As such, the increase in mechanical properties is similar to literature data relating to similar PMMA based nanocomposites in flexural strength and modulus, as well as microhardness and fracture toughness [19,20,30].

Table 1. Analysis of variance for flexural strength.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Factor	12	3010	250.850	27.42	0.000
Error	117	1070	9.148		
Total	129	4081			

Table 2. Analysis of variance for flexural modulus.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Factor	12	3.942	0.32848	9.49	0.000
Error	117	4.048	0.03460		
Total	129	7.990			

3.4. SEM Analysis of Fracture Surfaces

SEM images of the fracture surfaces of the specimens modified with 0 and 2% nanoparticles, obtained after bend testing, are shown in Figure 3. Dark circular phases scattered through the cross section are primary PMMA powder particles of 30–100 μm approximate size, actually the powder component of the PMMA material. Between them, lighter in colour is the polymerized material, initially being the liquid MMA, which was modified with 7 nm nanoparticles. This light grey phase is effectively a nanocomposite material in modified specimens or PMMA in the control specimen (specimen 0). Both specimens (0 and 2% nanoparticle) exhibited the fracture that was brittle in nature, which was expected. Its nature is clear after assessing the typical river marks present in both specimens, which are more pronounced in fracture surface of the specimen 0.

However, a more significant differences between the specimen without nanoparticles (Figure 3a) and the modified specimen (Figure 3b) can be observed. Namely, in specimen 0 (Figure 3a), a crack exhibits a mixed path regarding the propagation during fracture. The crack in specimen 0 propagates both through and around powder particles, as well as through the polymerized material. That means that there is an issue of powder-polymerized material bond, also indicating that there is a significant difference in strength between the polymerized lighter area in colour and powder spheres.

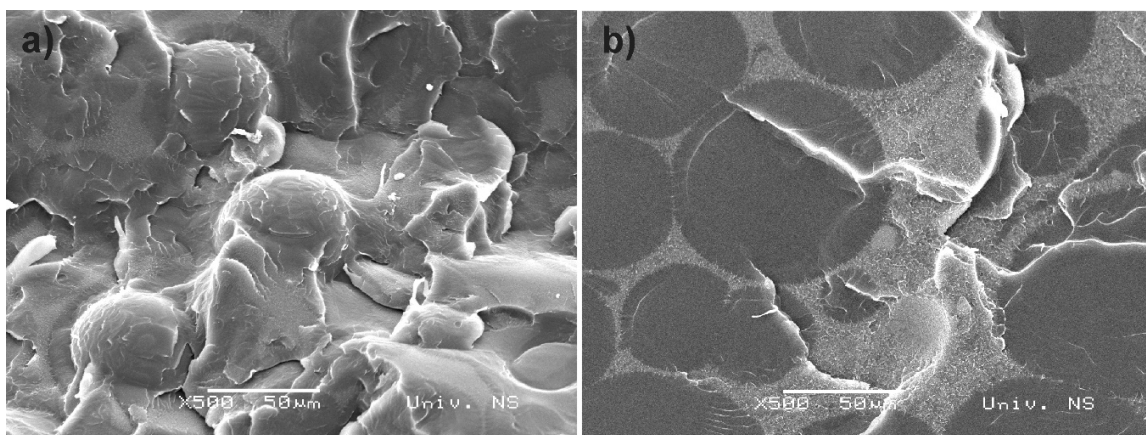


Figure 3. Fracture surfaces obtained after flexural testing of specimen 0 (a) and specimen modified with 2% nanosilica. A different crack propagation mode is revealed: predominantly through the nanocomposite between pre-polymerized powder particles (a) and through both pre-polymerized powder particles and polymerized nanosilica reinforced composite material (b).

A similar fracture morphology was found in autopolymerized PMMA [19,20], however, in this material, the crack propagates only through the polymerized light area. That means, the difference between the strength of the powder component and the polymerized PMMA around the powder

is even higher. This is due to the fact that in autopolymerized material, the radical polymerization process is much quicker, leaving less time for mixing and homogenization. The second effect is the lack of pressing of the material, which eliminates a significant portion of porosity. Finally, in hot polymerization, versus autopolymerization, that is also called cold polymerization, a higher amount of unconverted monomer is present, which does not contribute to mechanical properties. Quite contrary, the unconverted monomer acts as a microvoid representing a crack initiation site weakening the material. In the specimen modified with 2% nanosilica, a crack propagates through the PMMA powder and the polymerized nanocomposite, Figure 3b. This indicates a significantly stronger bond between the powder and polymerized nanocomposite around it, suggesting their strengths are similar to each other.

3.5. EDX Analysis of Agglomerates

EDX analysis of the certain phases observed on the fracture surface of the specimen modified with 2% is shown in Figure 4, along with the spectrum obtained in Table 3. Clusters of agglomerates were observed on the fracture surface, Figure 4. This indicates that agglomerates tend to cluster themselves together during fabrication process of the modified PMMA. On the other hand, it can be observed that these clusters consist of agglomerates and that these agglomerates have a similar size to those found by zeta sizer for this specimen modified with 2% nanosilica.

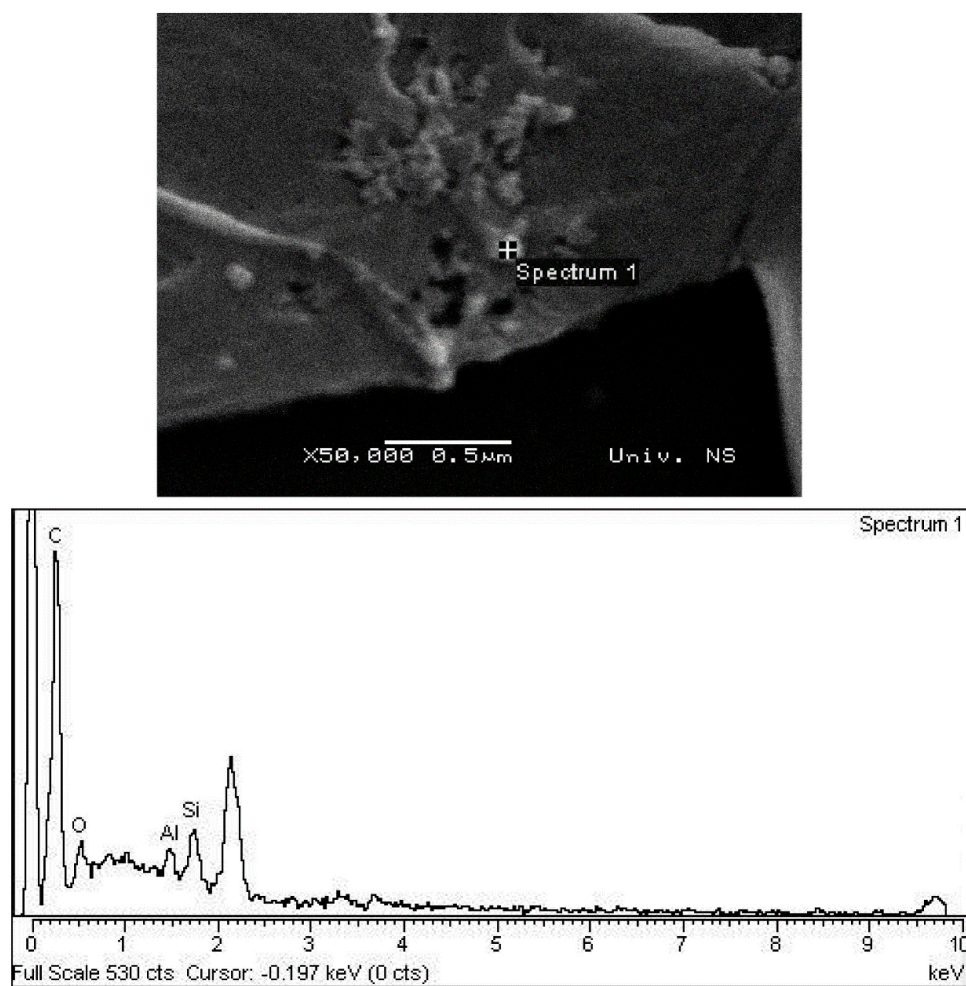


Figure 4. SEM images of fracture surface with a cluster of agglomerates (specimen modified with 2% nanoparticles) and EDX spectrum of particles. The analyzed spectrum contains Si and O, indicating that the point of interest is SiO_2 cluster of particles.

Table 3. Quantitative results of EDX analysis in wt.%.

Element	wt.%
C	73.07
O	22.11
Al	1.28
Si	3.54

EDX analysis reveals that this area contains silicon and oxygen, indicators of SiO₂. Although the content of Silicon is relatively low, EDX analysis analyses the material in depth, so a relatively large amount of PMMA is analyzed.

3.6. The Application of the Rule of Mixtures

The rule of mixtures (ROM) and the inverse rule of mixtures (IROM) are two common ways to predict the properties of composite materials in general, as shown in Equations (4) and (5) respectively [31]:

$$E_{\text{ROM}} = f_m E_m + f_r E_r \quad (4)$$

$$1/E_{\text{IROM}} = f_m/E_m + f_r/E_r \quad (5)$$

where E_{ROM} is the modulus of elasticity obtained by the ROM; E_{IROM} is the modulus of elasticity obtained by the IROM [GPa]; f_m is the volume fraction of the matrix; E_m is the modulus of elasticity of the matrix; f_r is the volume fraction of the reinforcement and E_r is the modulus of elasticity of the reinforcement.

The effectiveness of nanoparticle reinforcement can also be assessed by ROM and IROM. Modulus of elasticity of 71 GPa for SiO₂ was used for nanoparticles [32], while the unmodified PMMA (0% nanoparticles) was used for the matrix.

The calculated theoretical rule of mixtures and inverse rule of mixtures, as well as experimentally obtained values in this study are presented in Table 4. Table 4 reveals that elastic moduli of the specimens modified with 0.02, 0.05, 0.1, 0.2, 0.5 and 0.7% nanoparticles are higher than the moduli calculated in accordance to the ROM, with the study to rule of mixtures ratio higher than 1. The maximum is reached at 0.05% nanoparticle content, which is also the highest value obtained in this study.

Table 4. Theoretical values calculated based on rule of mixtures (ROM) and inverse rule of mixtures (IROM) compared to average elastic moduli obtained in this work.

	Modulus [GPa] for the Specimen Containing the Following Amount of Nanosilica [%]											
	0.02	0.05	0.1	0.2	0.5	0.7	1	1.5	2	2.5	3	5
ROM	2.25	2.26	2.27	2.30	2.40	2.46	2.56	2.72	2.87	3.03	3.19	3.82
IROM	2.24	2.24	2.24	2.24	2.25	2.25	2.26	2.26	2.26	2.27	2.27	2.29
This study	2.56	2.89	2.79	2.60	2.67	2.61	2.56	2.64	2.82	2.67	2.47	2.35
This study/ROM ratio	1.14	1.28	1.23	1.13	1.11	1.06	1.00	0.97	0.98	0.88	0.77	0.61

As the specimen nanoparticle content increases, the ratio drops, reaching the minimum at 5% nanoparticle content (ratio of 0.61). Furthermore, in the specimens having the highest concentrations of nanoparticles (3 and 5%), nanoparticles behave as particulate composite material fillers, since the moduli values approach those obtained by the IROM [31].

3.7. Results of Cytotoxicity

The influence of nanoparticle content and days of elution of the viability of L929 mouse fibroblasts and MRC 5 human lung fibroblasts is presented in Figure 4. Viabilities are the highest in specimens

after the shortest time of elution. The lowest cytotoxicity (the highest viability) was obtained in unmodified specimens (0%), while standard deviations are the lowest in these specimens as well. This is a similar trend to mechanical properties, where standard deviations were obtained in the specimens containing nanoparticles.

If the results obtained with two different cell lines used in this study are compared, it can be seen that trends are similar. However, it can be observed that MRC human lung fibroblasts are slightly more sensitive than L929 mouse fibroblasts, which is due to differences that exist between species. Results gained from human cell culture give more relevant results.

Biocompatibility tests based on mouse and human lung fibroblasts reveal a notable increase in cytotoxicity as the amount of nanoparticles in specimens increase. On the basis of presented results in Figure 5 and relevant standards [33,34], the nanoparticle content of up to 2% can be considered non-cytotoxic, as the cell viability reduction is 30% or less. That means that increasing nanoparticle content is both cytotoxic and non-beneficial from the point of view of mechanical properties.

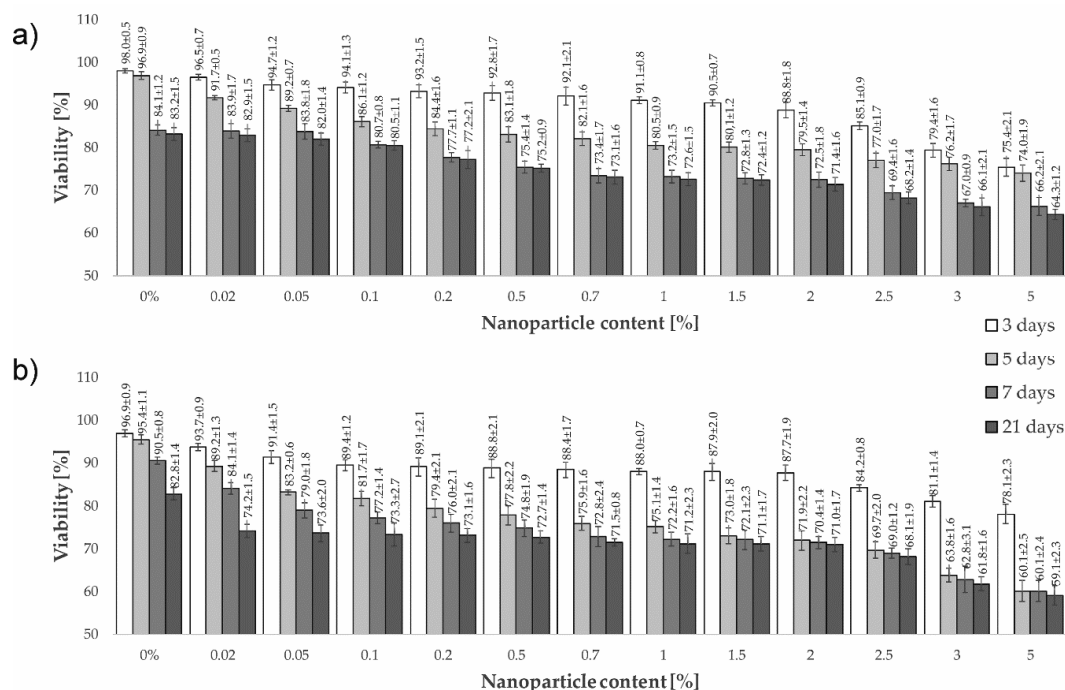


Figure 5. The influence of nanoparticle content and days of elution on the viability of L929 mouse fibroblasts (a) and MRC 5 human lung fibroblasts (b).

This proves to be highly selective, leaving 0.05% nanoparticle specimens as the optimal of all those tested in this study.

4. Conclusions

In this study, PMMA denture reline resins were modified with nanosilica treated with hexamethyldisilazane (HMDS) providing hydrophobic properties were tested in flexural strength and modulus, as well as cytotoxicity. Based on presented results and limitations in this study, the following conclusions can be established:

- The addition of nanoparticles is beneficial to an increased flexural strength and modulus of elasticity of hot polymerized PMMA.
- The increase in flexural strengths is statistically significant in relation to control group of specimens, except for specimens reinforced with 3% nanoparticles.
- In elastic moduli, all specimens exhibited statistically significant increase, except of the specimens reinforced with 3 and 5% nanoparticles.

- Mechanical properties are in good agreement with zeta sizer results, with the highest strength and modulus closely corresponding to the highest amount of smaller particles in the liquid phase.
- The size of detected particles in all modified specimens is considerably larger than the nominal nanoparticle size, indicating agglomeration occurred in all specimens.
- The highest flexural strength and modulus were obtained with the addition of 0.05% of nanoparticles.
- The addition of nanoparticles increases standard deviations of flexural strength and modulus.
- Specimens suffered a brittle fracture mode.
- The most efficient nanoparticle addition is 0.05%, with the average value of moduli higher than those the rule of mixtures suggests.
- The highest nanoparticle contents of 3% and 5% behave like fillers to the polymer material, providing the moduli that are closer to the inverse rule of mixtures.
- While an increase in nanoparticle content increases the material cytotoxicity, with specimens containing up to 2% of nanosilica it can be considered as non-cytotoxic.

It can be said that the most efficient and optimal 7 nm hydrophobic nanosilica content into hot polymerized PMMA is 0.05%. This loading provides the most convenient particle distribution in the liquid phase, the highest mechanical properties in terms of flexural strength and modulus, and can be considered as non-cytotoxic, by using both mouse and human lung fibroblasts.

Author Contributions: Conceptualization, S.B. and T.P.; investigation, M.P.; writing—original draft preparation, S.B.; writing—review and editing, D.D.K., T.P., and I.G.; funding acquisition, B.M. and J.L.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work is self-funded by the authors.

Acknowledgments: This research was supported by the Department of Production Engineering, Faculty of Technical Sciences Novi Sad Serbia, entitled “Modern technologies in materials science and welding technology”.

Conflicts of Interest: The authors declare no conflict of interest.

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