Preliminary Studies on Citotoxicity and Genotoxicity Assessment of the PMMA-TiO_2 Nanocompozites for Stereolithographic Complete Dentures Manufacturing

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The aim of the present study was to characterize and preliminary assess the cytotoxicity and genotoxicity of different PMMA-TiO_2 nanocomposites suitable to be used for two-parts stereolithographic complete denture manufacturing. In order to improve mechanical and antibacterial characteristics of the materials for 3D printing dentures, two matrix materials consisting in commercially available solutions of poly(methyl methacrylate) (PMMA), referred as dent-PMMA respectively base-PMMA, have been doped with different amount of TiO_2 nanoparticles: 0.4, 0.6, 1.0, and 4.0% by weight. The obtained nanocomposites were structural characterized and assessed for antibacterial activity, cytotoxicity and genotoxicity. The newly obtained nanocomposites presented a good inhibitory action against the considered bacterial species-Staphylococcus aureus, good biocompatibility under 4.0% by weight. The results of the preliminary studies on cytotoxicity and genotoxicity assessment recommends the use of 0.4% TiO_2 dent-PMMA and base-PMMA for CAD/CAM additive manufacturing of two-parts complete dentures/overdentures.

Keywords: TiO_2 - PMMA nanocomposites, 3D printing, cytotoxicity, genotoxicity, antibacterial activity, structural characterization

Biocompatibility, defined as the properties of materials being biologically compatible without causing local or systemic responses of a living systems or tissues, is the most important requirement for all the materials used in the oral cavity [1].

Poly(methyl methacrylate) (PMMA), among the polymer frequently used in dentistry, primarily for dentures and temporary crowns, individual impression trays, orthodontic devices or oral and maxillofacial appliances, possesses satisfactory biomechanical and esthetical properties [2,3]. For the common usage as denture base material and artificial teeth, PMMAs are subject to abrasion, accommodate pathogenic settlements of bacteria and release substances from the resinous matrix due to incomplete polymerization or resin degradation, causing adverse effects on surrounding buccal tissues. The release of methacrylic monomers together with compounds of the polymerization system from dental composites has been considered as a source of a wide variety of adverse biological reactions, which include local and systemic toxicity, allergic and estrogenic effects [4]. To enhance the mechanical and antibacterial properties of denture resins, many additives have been suggested such as fibers, fillers, or nanofillers [5-7].

One of the ongoing problems for the scientists and biomedical workers fighting against infectious diseases is the development of bacterial resistance. Nanotechnology offers a huge potential in biomedicine including the use of nanoparticles (NPs) as biocides. Many of these NPs are composed of heavy metals or metal oxides such as silver, gold, zinc, titanium dioxide, and zinc oxide [8,9].

Titanium dioxide (TiO_2) NPs, have been demonstrated to be an effective multifunctional material [6,10] with photocatalytic activities including killing bacteria and viruses, excellent mechanical properties, high degree of biocompatibility and low cost, being the fourth most abundant metal on earth [11,12]. All these characteristic, especially the antibacterial properties, recommended TiO_2 NPs as an ideal additive to enhance the performance of polymeric materials [6].

However, due to different characteristics of the NPs comparing to the materials in their original states, cytotoxicity and genotoxicity of the nanocomposites obtained are mandatory in order to be suitable for dental usage.

The aim of the present study was to characterize and preliminary assess the cytotoxicity and genotoxicity of different PMMA-TiO_2 nanocomposites suitable for stereolithographic complete denture manufacturing.

Experimental part

Materials

Two matrix materials consisting in commercially available solutions of poly(methylmethacrylate) (PMMA): E-Dent 100 and E-Denture (EnvisionTec GmbH, Germany) for 3 D printing have been used. Both solutions are recommended for temporary dental devices manufacturing, and will be further referred as dent-PMMA respectively base-PMMA. The inorganic filler added to these materials has been TiO_2 nanoparticles (anatase form, Sigma-Aldrich GmbH, Germany). The corresponding composite mixtures have been obtained through different additions of TiO_2 nanoparticles: 0.4, 0.6, 1.0, and 4.0% by

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weight, according to the procedure presented elsewhere [5,6,13].

Methods for characterizing the nanocomposite materials obtained

Structural characterization

Scanning electron microscopy (SEM) and the elemental distribution (EDX) have been applied for the nano-composites structural studies using an Oxford Instruments equipment. Also, the identification of the main active functional groups and the fingertip area has been performed through Fourier Transform Infrared - spectroscopy analysis (FT-IR) on Bruker Tensor 27 equipment.

Antibacterial activity

The antibacterial activity for dent–PMMA nano-composites has been presented elsewhere [6] and for the base-PMMA nanocomposites the antimicrobial activity against *Staphylococcus aureus* (ATCC 25923) was evaluated in aqueous suspension according to the following procedure: Stock cultures of *S. aureus* (ATCC 25923) were transferred into tryptic soy broth (TSB, Lab M, Bury, UK) and incubated at 37°C for 24 h. Then, cultures were centrifuged at 3.600 g for 10 min at 5° C, washed twice, suspended in Sorensen's phosphate buffer (SPB, 0.3 mM KH_2PO_4, *p*H 6.8) and the cell density was adjusted to a 0.5 McFarland turbidity standard approximately 1.5×10^8 colony forming units (CFU)/mL. These suspensions were further diluted up to the working concentration of about 1.5×10^5 CFU/mL.

Antibacterial activity was quantitatively evaluated under dynamic contact conditions in agreement with ASTM E2149-13a standard [14]. According to the above-mentioned standard, each material type sterilized by UV treatment discs (~ 0.5 mg) were added to screw cap tubes having 1 mL of working bacterial suspension. The tubes were then positioned in an orbital shaker and shaken at 220 rpm for 90 min. The number of viable bacteria was determined by plate count technique for initial time (time 0) and after exposure. Suspensions and additional decimal dilutions up to 10^{-3} were plated on Nutrient Agar (NA, Lab M) and incubated for 48 h at 37 °C followed by counting of the colonies to represent as CFU/mL. The experiments were performed in 3 replicates. The antibacterial activity of the samples expressed as percent reduction (R) values was calculated using the following formula:

$$R = [(A-B) / A] \times 100 \quad (1)$$

where A is the initial (time 0) counts (CFU/mL), and B is the counts (CFU/mL) after 90 min of contact.

Biocompatibility and Cytotoxicity assessment

The cytotoxicity, as an important aspect for dental polymeric nanocomposites, has been assessed as follows:

Assessment for dent-PMMA nanocomposites

Extract test [15, 16]: 0.2 g samples from dent-PMMA (without TiO_2 NPs – Null sample), dent-PMMA + 1.0% TiO_2 nanoparticles and dent-PMMA + 4.0% TiO_2 nanoparticles were separately extracted in 1 mL Minimum Essential Medium (MEM) at 37°C for 72 h. Then the extracts were filtered through 0.22 micron filters for sterilization followed by determination of cytotoxicity through MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay. The enzymatic reduction of MTT to MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase, depending on mitochondrial respiration, therefore, indirectly assessing the cellular energy capacity of a cell. The sample only containing cell and medium was accepted as negative control. The viability of the cells in the medium that also contains extracts was compared to negative control. Phenol was used as positive control.

The fibroblast cell line L929 were plated on 96 well plates at a seeding density of 10^4 - 10^5 cells/mL and allowed to attach overnight at 37°C. The samples were dissolved in 1% dimethyl sulfoxide (DMSO) and added to the wells at a concentration of 1.5 mg/mL in 3 replicas. As negative controls only medium has been added, while phenol was used as a positive control and 1% DMSO as DMSO control. Different PMMA samples and control groups were seeded for 24 h. MTT assay was used to determine the cytotoxicity and proliferation. Optical density was measured at 570 nm with Elisa reader.

Assessment for base-PMMA nanocomposites

The L929 cell line was used for determination of the cytotoxicity of the nano-polymer. In this regard, the cells were cultured in DMEM/F-12 (Dulbecco's Modified Eagle *Medium*/Nutrient Mixture F-12) medium (supplemented with 10% fetal bovine serum and penicillium-streptomycin) and incubated at 37°C in 5% CO_2. After 3-4 days, the cultured cells were detached by trypsinization and counted with a hemocytometer.

The L929 cells were seeded 10^4 cells/well in 96-well flat-bottom microplates with 100µL of medium. Cells were incubated for 24 h at 37°C in order to attach to the well bottoms. Polymeric material samples cut same size were placed in well containing the cell and incubated for another 24 h.

The media containing cell culture were then aspirated and 100 µL of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) solution in fresh medium was added to the wells at a concentration of 0.5 mg/mL (with 7.5 µg/mL phenazine methosulfate). The plates were incubated for 4 h at 37°C and optical density was measured at 450 nm with a multi-plate reader (Thermo Labsystems Multiskan Ascent 354 Microplate Photometer).

Preliminary genotoxicity assessment of nanocomposites

In vitro micronucleus (MNvit) test was conducted in compliance with OECD 487 [17] recommendations. Human lymphocyte cultures were initiated. Peripheral blood was taken from a middle-aged, healthy female who had not been recently exposed to genotoxic agents (chemical substances, ionising radiations, bacterial/viral infections). Peripheral blood was collected using heparin-based anticoagulant. About 0.5 mL of heparinised blood was transferred in a tube containing 10 mL supplemented PB-MAX (GIBCO). The culture was incubated at 37°C for 24h. The base-PMMA and PMMA-TiO_2 (TiO_2 0.4%) were introduced in the human lymphocytes culture 24 h after the culture was initiated. They were previously sterilised being exposed to UV for 15 min. Three concentrations were tested: 1.25 mg/mL 2.5 mg/mL and 5 mg/mL for each type of a sample and a sample free of nanocomposites was used as control sample. Each sample was in duplicate. The cultures were sacrificed 72h after nanocomposites was added, which was enough time for the cells to undergo several cell division rounds so that potential DNA damage in the form of micronuclei in interphase cells could be identified. The culture was developed without adding colchicine (actine polymerisation inhibitor). In the next steps the cultures were sacrificed by hypotonization using K citrate solution, and cells were fixed in 3:1 methanol/ acetic acid solution. Microscope slides were prepared, stained using Giemsa solutions. An Olympus BX40 microscope was used to analyse the slides.

The 3D printed complete denture

The obtained materials (PMMA-TiO_2 nanocomposite) have been utilized for a two-piece stereolithographic prototype for complete denture manufactured with the use of EnvisonTEC Perfactory®3D printer (Gladbeck, Germany). The two-piece denture was design using 3Shape CAD software (Copenhagen, Denmark). Each of the two parts were separately printed: for denture base manufacturing nano TiO_2 base-PMMA (with pink color) was used and artificial teeth were obtained from nano TiO_2 dent-PMMA color A2. The detailed manufacturing technique and post processing procedure were described elsewhere [3,13].

Results and discussions

Structural characterization

SEM images obtained from the nanocomposites used for denture teeth manufacturing (dent-PMMA) reveals an increased roughness surface with a higher concentration of TiO_2 nanoparticles. This surface roughness is due to the aggregations of PMMA and TiO_2 (fig. 1).

Figure 2 presents the morphology of two nano-composites: base-PMMA with 0.4% TiO_2 and 0.6% TiO_2 by weigh nanoparticles inclusions. The morphology of the surface is smoother compared to the dent-PMMA composites. The elemental mapping analysis (EDX) highlights the presence of the base-PMMA components (fig. 3).

It is easily noticed that the TiO_2 is the only filler of the base-PMMA matrix, the structure and morphology aspects being cleaner compared with those characterizing the dent-PMMA.

In figure 4, FT-IR spectrum recorded for dent-PMMA nanocomposites obtained with 0.4% addition of TiO_2 nanoparticles is presented. Also, (fig.4b). introduces the FT-IR spectrum of the denture resin which was treated under UV radiation at 364 nm for 5 min before analysis. It could be observed that there are two weak peaks at 3,420 cm^{-1} and 1,602 cm^{-1} showing the -OH group stretching and bending vibrations, respectively. The presence of these bands most probably comes from the adsorbed water molecules [18].

The two bands recorded at 2,980 cm^{-1} and 2,910 cm^{-1} can be assigned to the C–H bond stretching vibrations of from $-CH_3$ and $-CH_2-$ groups, respectively. The band at around 1,740 cm^{-1} shows the C=O stretching of acrylate carboxyl group. The band at 1,458 cm^{-1} can be attributed

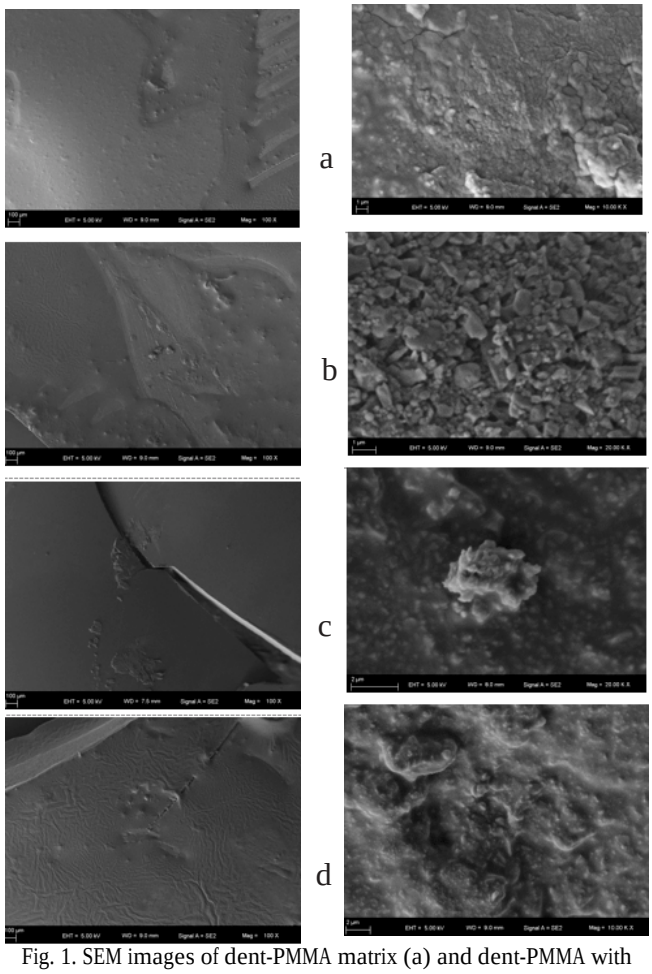


Fig. 1. SEM images of dent-PMMA matrix (a) and dent-PMMA with incorporated nanoparticles: 0.4 TiO_2 (b), 1.0% TiO_2 (c) and 4.0% TiO_2 (d)

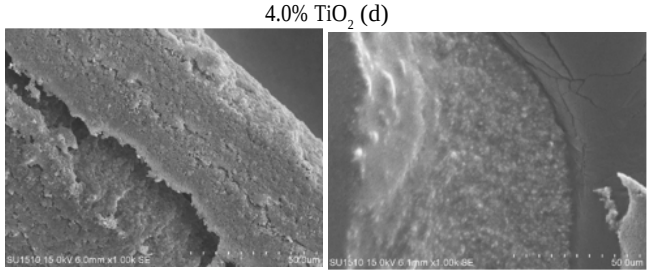
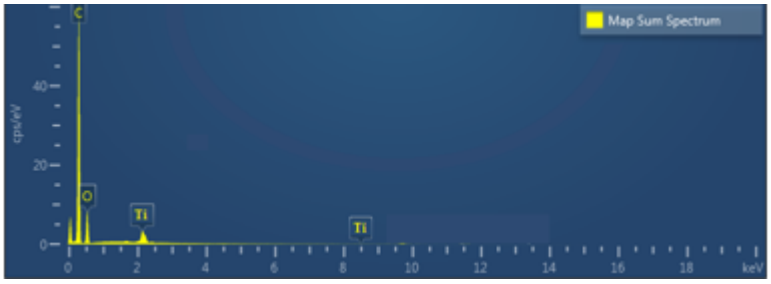
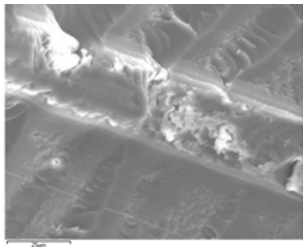


Fig. 2. SEM images of base-PMMA nanocomposites with incorporated 0.4 (a) nano-TiO_2, and 0.6% nano-TiO_2 (b)





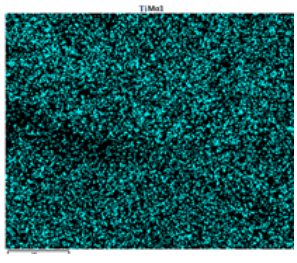


Fig. 3. Elements distribution for base-PMMA nanocomposites with 0.6% nano-TiO_2 incorporated

Map Sun Spectrum				
Element	**Line Type**	**Weight %**	**Weight % sigma**	**Atomic %**
c	K series	69.16	0.22	79.05
o	K series	23.85	0.20	20.46
Ti	M series	6.99	0.15	0.49
Total		100.00		100.00

to the bending vibration of the C-H bonds of the -CH_3 group. The bands at 1,372 and 758 cm^{-1} are characteristic to the α-methyl group vibrations. The bands at 1,040 cm^{-1} and 829 cm^{-1} put in evidence the absorption vibrations of PMMA skeleton. No significant, differences were found between FTIR spectra when nano-TiO_2 has been added and the Ti-O-C bond shifted towards 750 cm^{-1} could be identified (fig. 4).

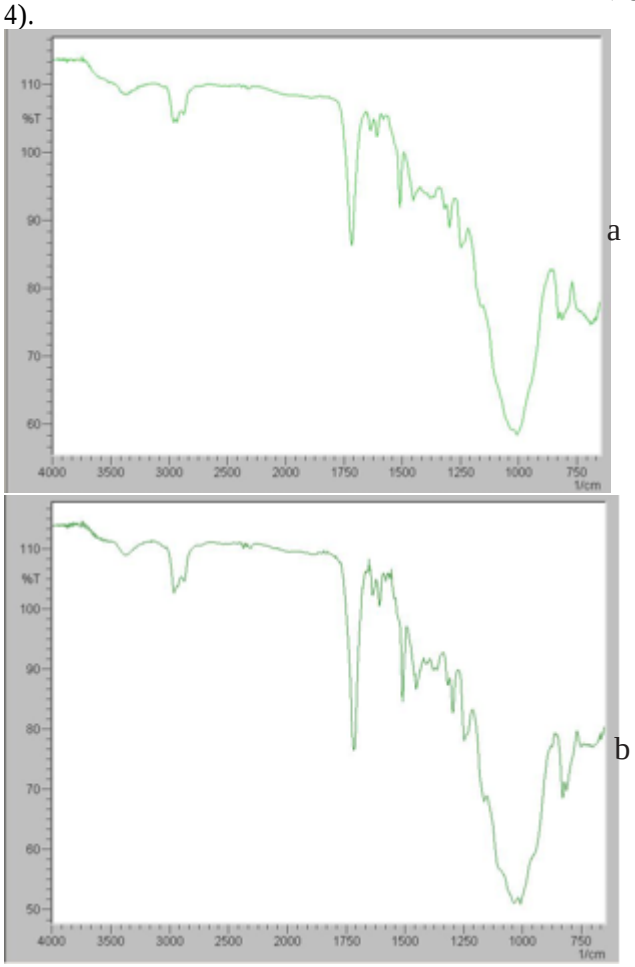


Fig. 4. FT-IR spectra for dent-PMMA 0.4% nanocomposite (a); UV irradiated dent –PMMA nanocomposite (b)

The FT-IR spectra for base-PMMA presents two weak peaks at 3,385 cm^{-1} and 1,608cm^{-1} showing -OH group stretching and bending vibrations, respectively. The groups -OH most probably comes from the adsorbed water molecules [18]. The bands around 2,960 cm^{-1} can be assigned to the C-H bond stretching vibrations of the -CH_3 and -CH_2- groups. The band at around 1,716 cm^{-1} shows the C=O stretching of acrylate carboxyl group. The band at 1,453 cm^{-1} can be attributed to the bending vibration of the C-H bonds of the -CH_3 group. A neighboring peak to the right around 1,400 cm^{-1} could indicate the presence of C-O bond vibration. The bands at 1,383 and 748 cm^{-1} could be attributed to the α-methyl group vibrations. The bands around 1,030 cm^{-1} and 829 cm^{-1} are the characteristic absorption vibrations of PMMA.

There is a clear difference between the two PMMA varieties used as matrices for teeth and base for 3D printed complete dentures manufacturing. More peaks could be seen in the dent-PMMA graphs than their base-PMMA counterparts, especially towards the fingerprint region. Dent-PMMA spectra have a wide band structure between 1,200-850 cm^{-1} which probably hide the following band structures that can be seen in base-PMMA spectra: C-O vibration band at 1,161 cm^{-1}, and typical absorption bands of PMMA at 1,062 and 940 cm^{-1}.

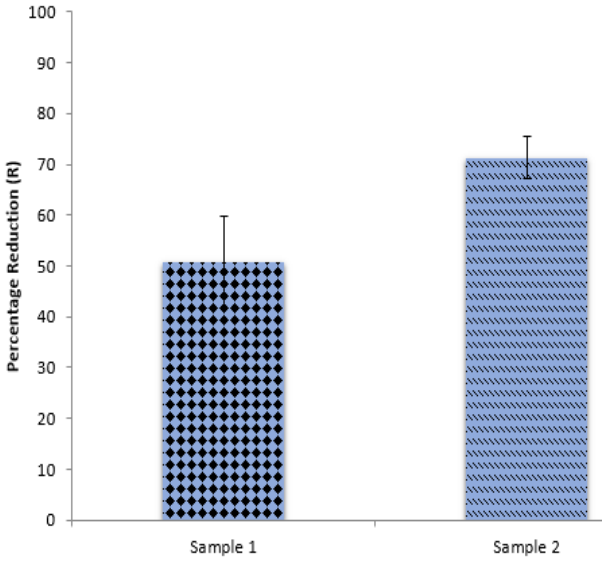


Fig. 5. Antibacterial activity of the base-PMMA samples against *S.aureus* in aqueous suspension, were Sample 1 is base-PMMA – 0.4 % TiO_2 and Sample 2 is base-PMMA (E-Denture) without nanoparticles inclusions. Error bars denote the standard deviation

Antibacterial activity

The antibacterial study performed on base-PMMA - TiO_2 nanoparticles composites presented a good inhibitory action against the considered bacterial species - *S.aureus*. The obtained results presented in figure 5 confirm previous studies [5,19] regarding the antimicrobial activity of the TiO_2 nanoparticles added to polymeric matrix. The two-tailed P-value equals 0.0227 and by conventional criteria ($P<0.05$), this difference is considered to be statistically significant.

Biocompatibility and Cytotoxicity assessment

The results of the biocompatibility and cytotoxicity assessment for both dent-PMMA and base-PMMA nanocomposites were encouraging.

Cytotoxicity assessment for dent-PMMA

According to Fig. 6 (Extract test – Experimental) the viability of the dent-PMMA (Null sample) was 80.688%.

The 1.0% nanocomposites and dent-PMMA can be acceptable as biocompatible since the viability percentage was over 70% (79.356%), while the 4.0% nanocomposite showed a certain level of cytotoxicity.

The mean optical density (OD) value of the negative control group treated with medium only was standardized as 100% alive and the OD570 values of test samples were compared to this value. As can be seen from figure 7, the

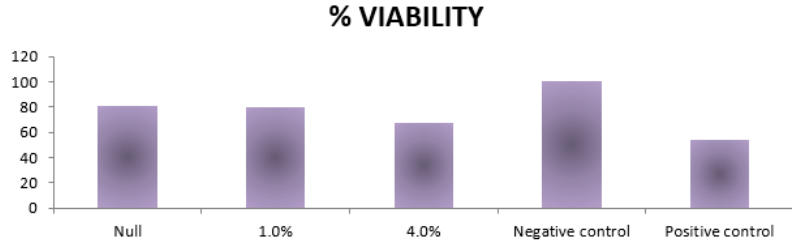


Fig. 6. Biocompatibility assessment for dent-PMMA nanocomposite

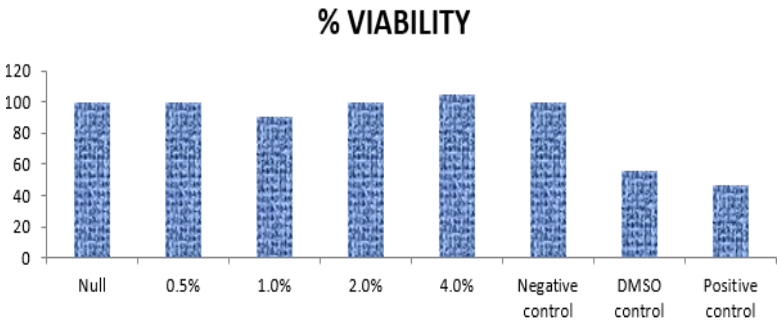


Fig. 7. Cytotoxicity assessment for dent-PMMA nanocomposites

viability of cells exposed to dent-PMMA polymer (Null) was 99.3%, while for 0.5% TiO_2 in PMMA polymer was 99.9%, 1.0% TiO_2 - PMMA nanocomposite was 90.0%, 2.0% TiO_2 - PMMA was 99.4% and for 4.0% TiO_2 - PMMA composite was 104.7%. According to grades of cytotoxicity [15] it is grade 1 and it is accepted as biocompatible. At this concentration, polymers showed proliferative effect on cell growth because DMSO control with 1% DMSO showed only 56% viability.

Cytotoxicity assessment for base-PMMA

For the base-PMMA nanocomposites the materials toxicity was assessed applying the XTT method (described in Experimental paragraph). The investigated material had no toxic effect on fibroblast cells (fig. 8).

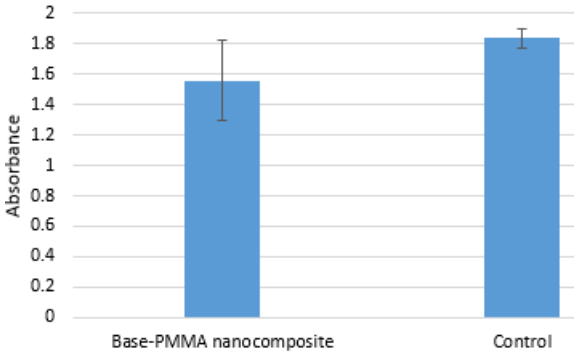


Fig. 8. Cytotoxicity assessment for base-PMMA nanocomposite (0.4% NPs TiO_2 added) as compared to Control (base-PMMA without NPs)

Preliminary genotoxicity assessment of nanocomposites

In order to preliminary analyze the genotoxic of nanocomposites (base-PMMA and PMMA-TiO_2 0.4%) in vitro Micronucleus Test was performed (MTvit) according to the procedure described in Experimental paragraph. The results are presented in table 1.

For the 5mg/mL concentration, the MN incidence could not be calculated as the genetic material was seriously damaged [20,21]. The concentration of 5 mg/mL was too high to allow for assessing the micronuclei incidence. The genotoxic capacity was determined by the high concentration and by the fact that the nanocomposites precipitated forming aggregates and directly binding to DNA.

The MN incidence analysis for 2.5 mg/mL concentration indicated a high degree of genotoxicity and the presence of lesions in the genetic material. Both 5 mg/mL and 2.5 mg/mL nanocomposite concentrations in culture medium were too high for the genotoxic analysis.

The nanocomposite concentration of 1.5 mg/mL in the culture medium resulted in a medium to low incidence of micronuclei compared to the negative control sample. However, this concentration favours the assessment of the genotoxic capacity. Compared to the negative control sample, a low DNA damage has been found, especially in PMMA-TiO_2 sample (fig. 9). The incorporated TiO_2 NPs in PMMA could stabilize the interactions between DNA and PMMA. Positively charged methyl groups from PMMA interacts with the negatively charged phosphate groups from DNA leading to ionic bonds that disrupts DNA structure. TiO_2 nanoparticles incorporated in PMMA could neutralize the methyl groups and consequently stabilize the nanocomposite, rendering it less reactive to the genetic material.

According to OEDC, *in vitro* micronucleus test (487 OEDC Guideline) it is recommended as basic test to assess and characterise the genotoxic capacity of chemical/ pharmaceutical agents. It is used to detect the micronuclei in the cytoplasm of interphase cells. Micronuclei are

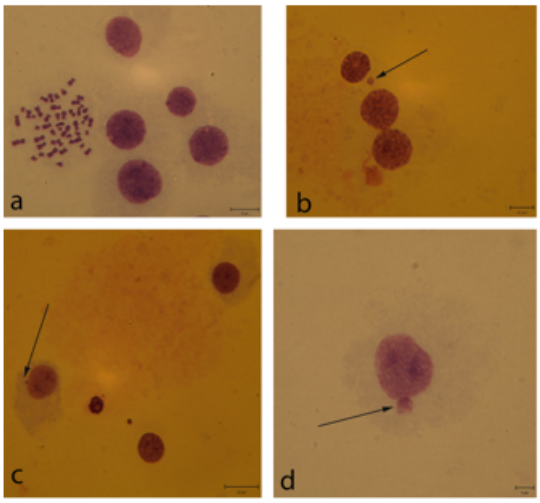


Fig. 9 MNvit analysis: normal metaphase and nuclei from negative control (a); micronucleus (arrow) from negative control (b); micronucleus (arrow) from base-PMMA (c); micronucleus (arrow) from PMMA-TiO_2 (d)

Nanocomposite type	**Nanocomposite concentration in culture medium**		
	1.5 mg/mL	2.5 mg/mL	5.0 mg/mL
PMMA-base	6.8‰	-	-
PMMA-TiO_2	5.8‰	-	-
Negative control	5.2‰		

Table 1
MICRONUCLEUS INCIDENCE OF BASE-PMMA AND PMMA-TiO_2 COMPARED TO NEGATIVE CONTROL

acentric (without centromere), chromosomal fragments or whole chromosomes unable to migrate to one of the two poles of the cell during anaphase Through *in vitro* micronucleus test (MNvit) the frequency of chromosomal damage as well as of the disturbance in the cell cycle progression caused by a chemical agent (which is tested) can be rapidly determined. This test has become more popular than the chromosomal aberrations test (CAAT) because it is more feasible, easy to conduct, rapid, and more sensitive in detecting subtle damage caused by aneugenic and clastogenic agents.

The results of the preliminary studies on cytotoxicity and genotoxicity assessment recommends the use of 0.4% TiO_2 dent-PMMA and base-PMMA for CAD/CAM additive manufacturing of two-parts complete dentures/ overdentures[22,23].

Conclusions

Dent-PMMA and base-PMMA 0.4% TiO_2 nano-composites showed excellent antibacterial activity, biocompatibility and cytotoxicity profiles, sustaining in this way their usage for two-parts 3D printed dentures/ overdentures manufacturing. The experimental work and investigations performed put in evidence the alterations induced by the presence of the inorganic nano-filler (TiO_2) leading to improved materials suitable for dental use.

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