NANO-MATERIALS IN DENTAL IMPLANTS - UNDERSTANDING COMPOSITION AND BIOFILM DYNAMICS

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The study showcases findings from an investigation into the chemical composition and morphology of nanocomposite materials employed in the production of dental implants. By utilizing clinical strains of microorganisms isolated from patients with implant infections (including *Bacteroides fragilis*, *Porphyromonas melaninogenica, Actinomyces spp., and Staphylococcus aureus*), the authors of the paper analyze the results of the dynamics of biofilm formation on the composite's surface. To minimize complications following surgical procedures in dentistry, the recommendations on the most advantageous research algorithm for composite materials are provided. The implications of these findings extend to the broader field of biomaterials, emphasizing the significance of a meticulous examination of the chemical and morphological attributes for enhancing the long-term success and safety of dental implants. This comprehensive approach addresses immediate concerns related to biofilm formation and provides a foundation for developing more resilient and biocompatible materials in dental prosthetics.

KEYWORDS

Nanopatterned surfaces, antimicrobial surfaces, nano-materials, nano-structured surfaces, 3D printing, bio-mimicked composite human bone implant joints, biomedical engineering, apatite-biopolymer composites

1 INTRODUCTION

With the advancement of computer-aided design and computer-aided manufacturing (CAD-CAM) technology [Bayarsaikhan 2022, Baba 2021] and additive manufacturing (AM) [Tahayeri 2018], 3D printing is actively used in dentistry for the creation of both fixed and removable dental prostheses [Jang 2023, Shim 2020]. These technologies streamline the prosthetic manufacturing process, allowing dentists to obtain more precise and individualized solutions for each patient. For instance, with CAD-CAM, dental models can be created based on intraoral scanning, and prostheses can be fabricated directly from these models.

Composite polymer materials are currently predominantly used for fabricating temporary and permanent dental prostheses. These materials possess high strength and can be easily modified to achieve the desired aesthetic and functional properties. Moreover, they are typically less traumatic to tissues compared to other materials. Additive manufacturing encompasses technologies such as stereolithography (SLA) [Barszczewska-Rybarek 2019], digital light projection, fused deposition modeling, selective laser sintering, inkjet printing, photopolymer inkjet printing, and powder bed fusion [Pavan Kalyan 2022]. These technologies enable the fabrication of prostheses from various materials, including polymers, composites, ceramics, and metal alloys [Tartaglia 2021, Lim 2022, Bichu 2023]. Each of these technologies has its unique advantages and limitations, allowing dentists to choose the optimal manufacturing method based on specific requirements for the prostheses.

Among the various methods in digital dentistry, cube-based polymerization methods such as digital light processing (DLP) and stereolithography are widely utilized. Polymerization of photopolymer resin is a complex procedure that depends not only on the material characteristics but also on the properties of the curing machines. According to previous research, the degree of resin conversion printed by a 3D printer can be increased explicitly by factors such as oxygen inhibition, high wavelength of ultraviolet radiation, intensity of polymerization light, and temperature during post-polymerization reaction. The physical properties and biocompatibility of DLP resin must be clinically adequate for the long-term functioning of the prosthesis in the oral cavity. The hardness of the prosthesis base is essential for its resistance to surface abrasion, damage, and scratches during clinical use. DLP resin's degree of conversion (DC) and cytotoxicity are important considerations to avoid undesirable effects, as residual unpolymerized resin monomers can irritate the oral mucosa or damage cells [Jindal 2019].

Standard materials for making aligners include polyurethane polymers and polyethylene terephthalate glycol (PETG), which undergo a thermoforming process on physical models. Martina et al. [Martina 2019] and Alhendi et al. [Alhendi 2022] reported mild to moderate toxicity during testing of several transparent alignment systems, while Eliades et al. [Eliades 2009] found no evidence of cytotoxicity to human gingival fibroblasts after using Invisalign. Evaluating various brands, Martina et al. also demonstrated that the thermoforming process can increase the in vitro cytotoxic effect of different thermoplastic materials on primary human gingival fibroblasts. However, several parameters may influence the cytotoxicity of materials for 3D printing, including material composition, printing conditions (type and speed of the printer or orientation of the aligner on the working plate), and post-processing procedures (rinsing and post-curing) [Dyadyura 2017a, Pratsinis 2022, Wulff 2022].

The fluctuations in pH in the oral cavity, humidity, pressure, and temperature, in addition to the enzymatic activity of bacteria and saliva, can lead to the mechanical and chemical transformation of thermoplastic materials, which may result in the leaching of unreacted components [Yazdi 2023]. Therefore, the biocompatibility of aligner materials post-treatment is associated with the potential for alternative, non-microbial etiologies, peri-implantitis, including a reaction to the foreign body as a protective response against peri-implantitis. Furthermore, the implant's material, shape, and surface characteristics can be associated with an increased risk of peri-implantitis formation [Zaborowski 2007, Adamcik 2014, Svetlik 2014, Rimar 2016, Olejarova 2017 & 2021, Sedlackova 2017, Catlos 2018, Labun 2018, Gamec 2019, Kuznetsov 2019, Murcinkova 2019, Pollak 2019 & 2020, Straka 2021 & 2022, Vagaska 2021].

On the other hand, the components of dental implant material also play an essential role in the process of osseointegration. They may act as a source of biofilm formation [Alves 2022], which has a polymicrobial nature and a high degree of invasiveness [Dyadyura 2017b, Krenicky 2022, Vernon 2022]. Consequently, each stage of material processing can hurt its biocompatibility. In this regard, simple systems for quickly assessing the safety and biocompatibility of new implant materials deserve special attention. Single-celled organisms can be successfully used in universal rapid test systems to assess the safety of nanocomposites [Jurko 2011, Monkova 2013, Michalik 2014, Panda 2014, 2021 & 2022, Baron 2016, Mrkvica 2016, Hrebenyk 2017, Macala 2017, Balara 2018, Chaus 2018, Duplakova 2018, Pandova 2018 and 2020, Sukhodub 2018a,b, Flegner 2019 & 2020, Harnicarova 2019].

This study aimed to compare the dynamics of biofilm formation on the surface of the composite and to evaluate the levels of cytotoxicity of the material for 3D printing transparent aligners under two different post-curing conditions, using clinical strains of microorganisms isolated from patients with implant infections (*Bacteroides fragilis, Porphyromonas melaninogenica, Actinomyces spp., and Staphylococcus aureus*). The null hypothesis that post-curing procedures would not affect the relative cytotoxicity of materials for 3D printing was rejected. According to manufacturers' recommendations, post-polymerization is necessary to avoid the initial toxicity of the 3D printing material. Resins for 3D printing are highly cytotoxic before the printing process, and the level of cytotoxicity decreases significantly after post-polymerization procedures involving the removal of uncured resin.

2 MATERIALS AND METHODS

For the investigation of biofilm formation on dental implants, clinical strains of microorganisms isolated from patients with implant-associated infections were used: *Bacteroides fragilis, Porphyromonas melaninogenica, Actinomyces spp.*, and *Staphylococcus aureus*. Species identification of the clinical experimental bacterial strains was conducted using classical bacteriological methods. The clinical isolates of microorganisms had their initial adhesion indices determined on standardized human O (1)Rh+ erythrocytes using the Brill's method, which is a significant initial factor in any bacterial infection. All strains of microorganisms isolated from patients with implant-associated infections and used to form a combined biofilm exhibited a high level of adhesion (IAM ≥ 5.5 bacteria/erythrocyte). Sterile 40 mm diameter Petri dishes were used for biofilm formation. Four milliliters of Mueller-Hinton broth were added to each Petri dish containing the dental implant sample, followed by the addition of a daily mixture of clinical isolate test cultures. Incubation was carried out for 12 days at 37°C. After cultivation, the implant samples were washed three times with 0.01 M phosphate buffer. The samples were fixed with 2.0% glutaraldehyde in 0.1 M phosphate buffer for 60 minutes, dehydrated in ethyl alcohol with increasing concentrations (30%, 50%, 70%, 80%, 90%, and 100%), and then air-dried for 12 hours. The dehydrated samples were sprayed with aluminum using a spraying apparatus. Morphological analysis of biofilm formation on dental samples was performed using SEM (Sumy State University) on the 14th day. Microbiological examination of planktonic microflora was also conducted during the study. Microbiological examination of planktonic microflora was performed using classical methods to identify pure cultures using the Bergey classification scheme and determine quantitative indicators of this microbiota.

3 MATERIAL PREPARATION

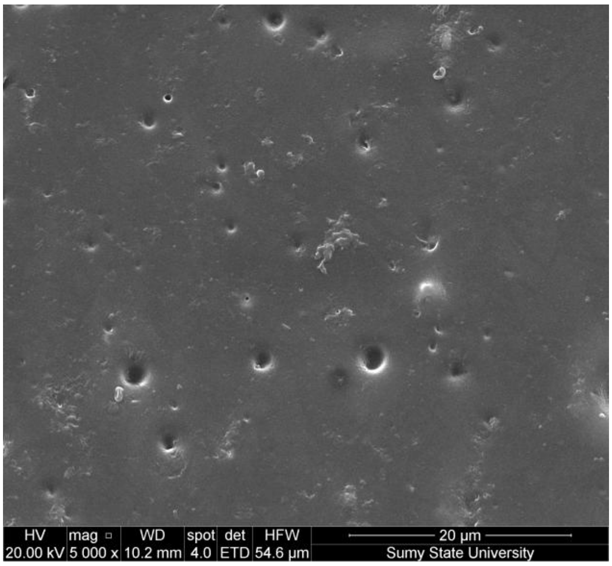
In this study, Tera Harz TC-85 resin, a photopolymer material for direct 3D printing of aligners provided by the Korean company Graphy Inc., was used to manufacture several copies of 3D-printed aligners of the same size and geometry. To assess the overall cytotoxicity of the material under different post-curing procedures and minimize the impact of additional variables on the cytotoxicity level, the aligners were printed using the same 3D printer. A DLP-based 3D printer (Max UV; Asiga) was used to manufacture the samples, with a light intensity of 7.50 mW/cm2, an exposure time of 2 seconds, and a wavelength of 385 nm. After printing, the samples were cleaned and processed with ethyl alcohol ultrasonication for 30 seconds according to the manufacturer's instructions. Supports were manually removed. Subsequently, all samples were post-cured using a UV-based polymerization system (Tera Harz Cure; Graphy) and under various atmospheric conditions by altering two different post-processing parameters: post-polymerization atmosphere (PPA, air, or nitrogen) and post-polymerization time (PPT, 5, 10, and 20 minutes). Three different types of samples were constructed using CAD software (Tinker CAD; Autodesk). Samples in the form of plates (65×10×3.3 ±0.2 mm) were prepared for qualitative analysis. A scanning electron microscope (SEO-SEM Inspect S50-B: energy-dispersive spectrometer AZtecOne with X-MaxN20 detector (manufacturer Oxford Instruments plc)) was used for quality analysis. Three samples were cleaned with DW ultrasonication and coated with Al. Images at 50× and 10,000× magnifications were obtained at an operating voltage of 15.00 kV and the working distance of 34.7 mm.

4 RESULTS

Observations of surface morphology were conducted using scanning electron microscopy (Fig. 1). Elemental analysis of the investigated sample was conducted using X-ray energy-dispersive spectroscopy (Fig. 2 and 3, Table 1).

In vitro investigation of biofilm formation processes by clinical isolates of microorganisms obtained from patients with implant-associated infections, as potential indicators of cytotoxicity, revealed that test samples of dental implants showed signs of initial biofilm formation processes (Fig. 2): primary microbial adhesion and initial stages of monolayer formation, presence of minor elements of biofilm extracellular matrix, absence of mature biofilm.

The microbiological study of the components of planktonic microflora revealed that it included all the strains used for biofilm synthesis under in vitro conditions, which were clinical isolates obtained from patients with implant-associated infections: *Bacteroides fragilis, Porphyromonas melaninogenica, Actinomyces spp.,* and *Staphylococcus aureus.* When determining the degree of colonization by planktonic microflora in the environment where biofilm cultivation was conducted, it was found that these indicators significantly increased to ≥ 10^5 CFU/ml ($p < 0.05$) on day 14 compared to the initial indicators of 10^2 CFU/ml in the environment. The presence of initial stages of biofilm formation and viable representatives of planktonic microflora indicate the absence of signs of cytotoxicity in the test samples of dental implants.



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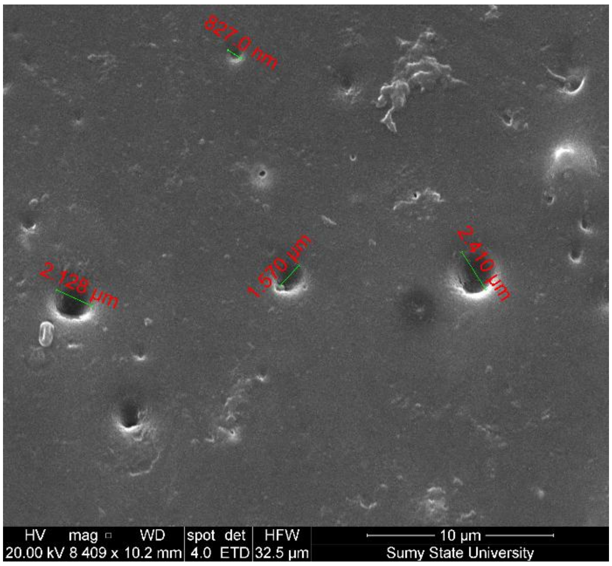

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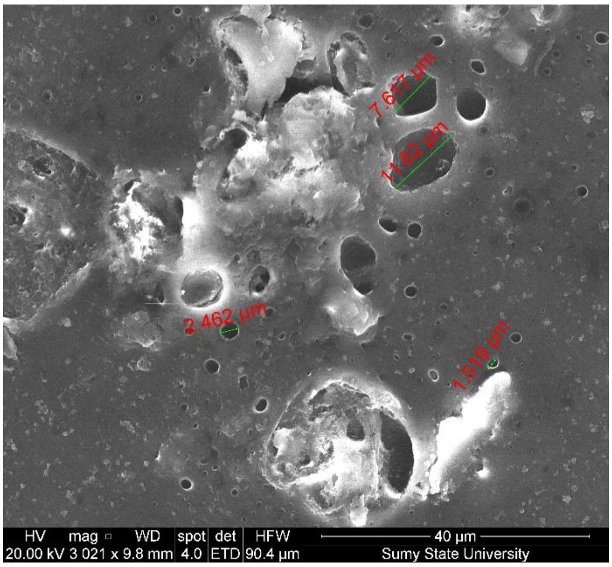

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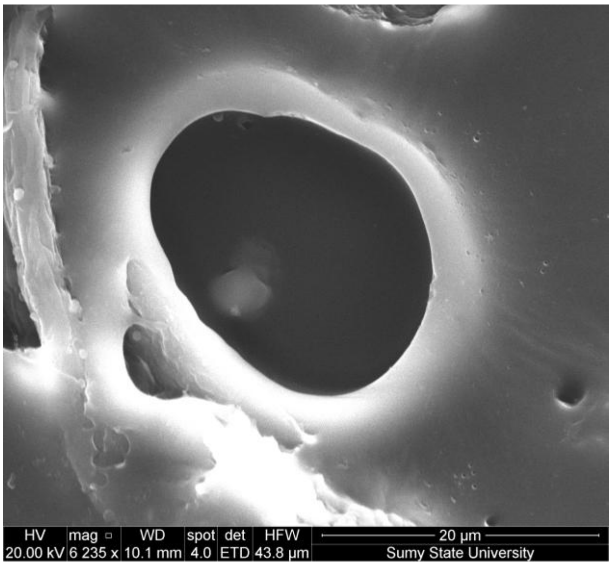

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Figure 1. Scanning electron microscopy images of resin samples (×50). (a) GP with cohesive failure inside the specimen, (b) NXT with cohesive failure inside the specimen, (c) MZ with cohesive failure inside the specimen, (d) VIPI with mixed failure, including cohesive failure inside the cement (white arrow) and adhesive failure (black arrow), (E) VIPI with adhesive failure

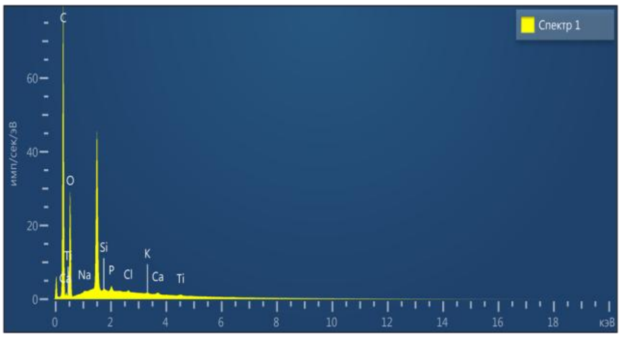


Figure 2. Results of analysis of energy dispersive X-ray spectroscopy

During the in vitro study of implant samples with a multispecies bacterial biofilm formed using all clinical isolates of test cultures, it was found that on the 14th day of the experiment, not only was there no complete biofilm on the surface of the implants (Fig. 5), but also the absence of multicellular bacterial aggregates that could exist for a long time and act as a "trigger" mechanism for biofilm formation. The obtained research results indicate that the investigated test samples of implants do not support such a process, which in turn prevents implant-associated infection formation at the human body level. Results from another series of in vitro studies on the quantitative and qualitative composition of planktonic microflora showed that in the environment in which cultivation of combined multispecies bacterial biofilm was performed for 14 days of incubation, all species of test cultures *Bacteroides fragilis, Porphyromonas melaninogenica, Actinomyces spp*., and *Staphylococcus aureus* were isolated in a significantly ($p < 0.05$) increasing amount. The absence of inhibitory action relative to the test cultures used to form the combined multispecies bacterial biofilm and the cytotoxic effect of the material of the investigational implants were observed.

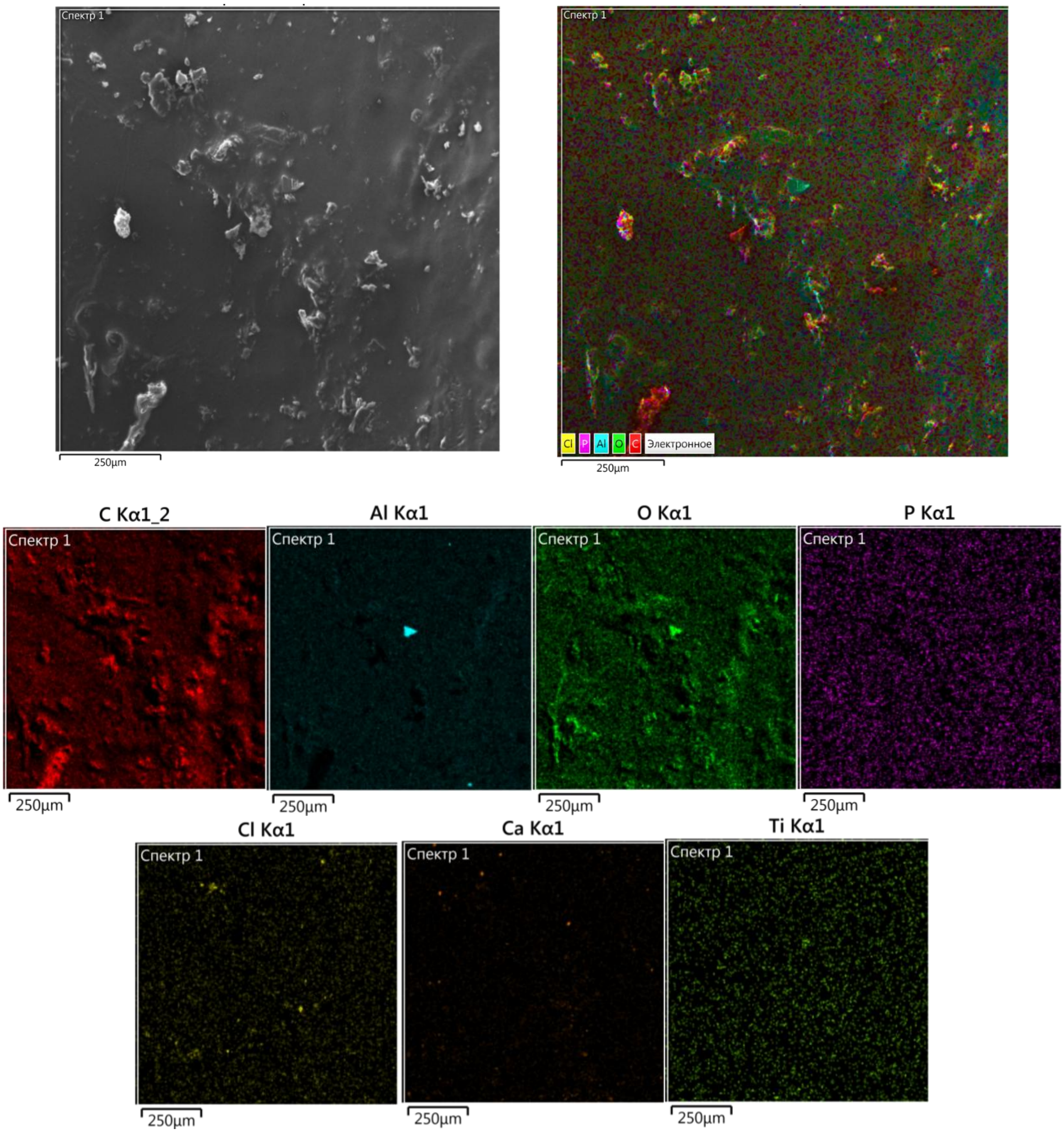
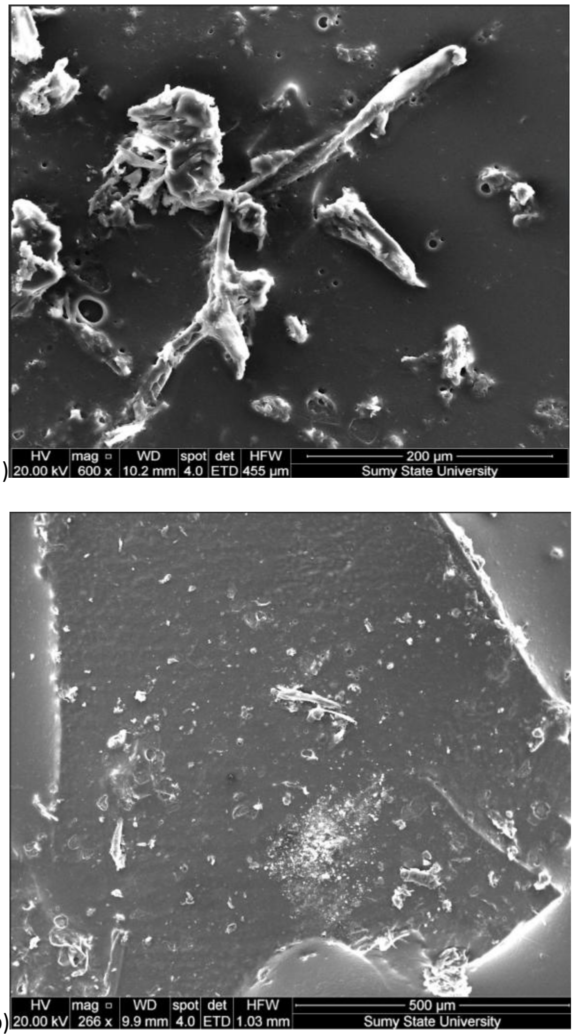


Figure 3. Image of the resulting 3D sample using the SEO-SEM Inspect S50-B Scanning Electron Microscope

Table 1. Residual monomer content of denture resin specimen according to PPA and PPT

Spectrum 1				
Element	**Line type**	**Weight %**	**Sigma weight %**	**Atom. %**
C	K-series	61.42	0.16	68.37
O	K-series	37.16	0.16	31.06
P	K-series	0.36	0.02	0.16
Cl	K-series	0.14	0.01	0.05
Ca	K-series	0.21	0.02	0.07
Si	K-series	0.26	0.01	0.12
Ti	K-series	0.20	0.02	0.05
K	K-series	0.12	0.02	0.04
Na	K-series	0.13	0.02	0.08
Total		100.00		100.00

During the in vitro study of implant samples with a multispecies bacterial biofilm formed using all clinical isolates of test cultures, it was found that on the 14th day of the experiment, not only was there no complete biofilm on the surface of the implants (Fig. 5), but also the absence of multicellular bacterial aggregates that could exist for a long time and act as a "trigger" mechanism for biofilm formation. The obtained research results indicate that the investigated test samples of implants do not support such a process, which in turn prevents implant-associated infection formation at the human body level. Results from another series of in vitro studies on the quantitative and qualitative composition of planktonic microflora showed that in the environment in which cultivation of combined multispecies bacterial biofilm was performed for 14 days of incubation, all species of test cultures *Bacteroides fragilis, Porphyromonas melaninogenica, Actinomyces spp*., and *Staphylococcus aureus* were isolated in a significantly ($p < 0.05$) increasing amount. The absence of inhibitory action relative to the test cultures used to form the combined multispecies bacterial biofilm and the cytotoxic effect of the material of the investigational implants were observed.



a)

b)

Figure 4. Results of cytomorphic analysis of biofilm composition on the surface of dental implants (SEM): a) Day 14: primary microbial adhesion; b) Initial stages of monolayer formation, presence of minor elements of biofilm extracellular matrix

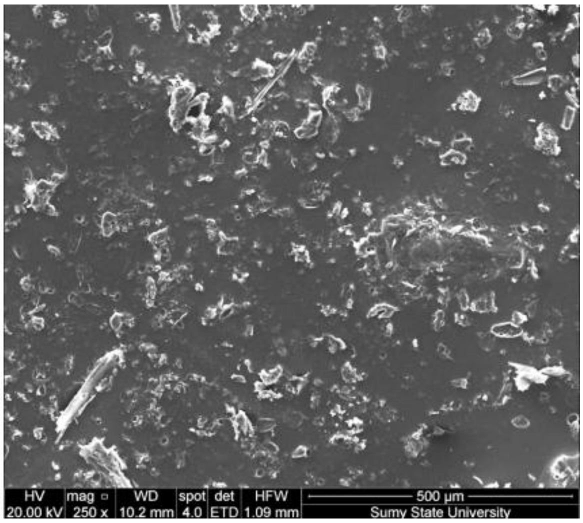


Figure 5. Results of cytomorphic analysis of the composition of the combined biofilm on the surface of dental implants (14 days of cultivation): absence of multicellular bacterial aggregates and complete biofilm

5 DISCUSSION

Most three-dimensional materials - printing resins are cured by free-radical polymerization using UV irradiation. When the material absorbs the wavelength of ultraviolet radiation, free radicals are formed, which induce the cross-linking of oligomers and monomers in the resin, leading to the formation of a cured polymer material.

Cytotoxicity is a general property of cell toxicity, which chemical factors can cause; the influence of other cells, such as NK or T-killer cells; and physical factors of the surrounding environment [Celik 2018]. Even in a healthy state, the oral cavity is colonized by microorganisms, which are also influenced by various factors [Chaturvedi 2018]. Thus, the oral cavity is an ecosystem with a balance between different types of microorganisms and the host organism's defense mechanisms [Hashim 2020]. The use of dental implants is one of the widespread methods for treating partial or complete edentulism; however, the implant's surface is also colonized by microorganisms [Shahabouee 2012]. Depending on the type of implant and the anatomical site of implantation, such microorganisms can cause implant-associated infections [Arciola 2018] as a result of biofilm formation on their surface.

It should be noted that the biofilm formation process is regulated by complex mechanisms of intercellular communication, such as Quorum Sensing (QS) [Vrynchanu 2019], which are also influenced by various factors, including the chemical composition of implants.

Considering that our study utilized a prolonged (14-day) method of biofilm formation under dynamic conditions (imitating the natural conditions of microorganism existence - clinical isolates isolated from patients with implant-associated infections with a high level of adhesion), the obtained results of studying the process of biofilm formation and the quantitative content of planktonic microflora prove the absence of cytotoxicity of the components of the test samples of dental implants. This is evidenced by the presence of viable cells of test cultures of microorganisms in the planktonic microflora in a significantly ($p < 0.05$) increased amount (CFU/ml of medium) compared to the initial values of the introduced bacterial cultures into the medium. Additionally, when studying the cytomorphological characteristics of the biofilm using SEM (Fig. 2) on the 14th day of biofilm synthesis, initial processes of its formation were detected: primary attachment of microorganisms to the surface, initial stages of monolayer formation, and the presence of minor elements of the biofilm extracellular matrix.

On the other hand, the widespread use and success of any dental material depend on preventing the formation of biofilms and infections mediated by their components [Hashim 2020]. Some authors' data on in vivo biofilm formation studies have shown that bacterial colonization can occur within 30 minutes of implant placement. Other studies indicate that oral streptococci dominate after dental implants are placed for 4 hours, and the number of anaerobic bacteria may increase within 48 hours [Shahabouee 2012]. The results of in vivo studies of biofilm formation indicate a high rate of realization of the first stage of biofilm formation on the surface of dental implants.

Analysis of the results of our in vitro studies of biofilm formation processes under dynamic conditions indicates that initial biofilm formation processes on test samples of dental implants occur only on the 14th day. Such delayed biofilm formation and maturation processes may protect against the cytotoxic effects of clinical strains at the organism level, which are accompanied by a decrease in functional activity or cell/tissue death at the implantation site.

6 CONCLUSIONS

Biological compatibility of dental materials is an essential issue as it indicates the material's ability to interact with the biological system without causing trauma associated with toxicity or harm to the oral cavity. Various post-polymerization procedures can influence the in vitro cytotoxicity of resin for 3D printing.

Our study, utilizing a 14-day method of biofilm formation under dynamic conditions mimicking natural microorganism existence, demonstrated the absence of cytotoxicity in test samples of dental implants. This was evidenced by the presence of viable cells of test microorganism cultures in significantly increased amounts compared to initial values, and cytomorphological analysis revealing initial biofilm formation processes on the 14th day. On the other hand, the success of dental materials relies on preventing biofilm formation and associated infections. In vivo studies have shown rapid biofilm colonization after implant placement, highlighting the importance of understanding and addressing biofilm formation dynamics. Our in vitro study revealed delayed biofilm formation processes on dental implant test samples, potentially providing protection against cytotoxic effects at the organism level. It has been recognized that aligners printed on a 3D printer and cured using Tera Harz Cure with a nitrogen generator are biologically compatible. Orthodontic laboratories and clinicians should adhere to the manufacturer's recommendations to avoid potential toxic effects during aligner treatment. Thus, due to the dependence of the cytotoxic properties of 3D-printed aligners on post-curing procedures, the evaluation of optimal conditions for post-polymerization of freshly 3D-printed material is warranted, considering a wide range of post-curing and curing conditions.

These findings underscore the significance of comprehensive evaluation and optimization of biofilm prevention strategies to enhance the success and safety of dental implant procedures.

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