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Biocompatibility of five occlusal splint materials produced by four fabrication methods: an in vitro comparative study

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Abstract

Background To evaluate the cytotoxicity, cell death, interleukin-6 (IL-6) release, and surface roughness of five occlusal splint materials produced by four different fabrication methods on L929 mouse fibroblasts.

Methods 240 disc-shaped specimens (15 × 2 mm) were fabricated from milled polymethyl methacrylate (PMMA), milled polyetheretherketone (PEEK), 3D-printed photopolymer resin, thermoformed polyethylene terephthalate glycol (PET-G), and auto-polymerizing PMMA. Extracts were prepared following ISO 10993-12 and collected on days 1, 3, and 7; at each time point the extracts of four specimens were pooled and applied to three replicate wells. Cell viability (XTT), total cell death (Annexin V-FITC/PI flow cytometry), IL-6 release (ELISA), and surface roughness (Ra, contact profilometer) were assessed. As the replicate wells were technical replicates of a single pooled extract, the biological data are reported descriptively; surface roughness was analyzed by one-way ANOVA with Tukey's HSD test ($p < 0.05$).

Results On day 7, the auto-polymerizing PMMA group showed the lowest cell viability (68.44 ± 3.48%) and was the only material to fall below the 70% threshold of ISO 10993-5. Cell viability remained above the 70% threshold in all other groups at all time points. Total cell death was low throughout (1.40–3.20%; untreated control 1.50%), with the highest day-7 values in the thermoformed PET-G (3.20 ± 0.30%) and auto-polymerizing PMMA (3.07 ± 0.86%) groups. IL-6 was below the limit of detection (18.75 pg/mL) in most conditions; only the auto-polymerizing PMMA group on day 7 yielded quantifiable IL-6, in two of three wells (33.78 and 42.55 pg/mL). The milled-PEEK and thermoformed PET-G groups showed the lowest Ra values, whereas the milled-PMMA group showed the highest ($p = 0.005$).

Conclusions In this in vitro extract model, the auto-polymerizing PMMA group was the only material falling below the 70% viability limit of ISO 10993-5 and the only one with quantifiable IL-6 on day 7. All other groups remained above this threshold throughout, and total cell death remained low in all groups. The milled and printed materials evaluated in the present study may therefore represent biologically more favorable options than the auto-polymerizing PMMA group. These findings are descriptive, based on a single pooled extract per condition, and apply only to the specific products tested.

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Keywords Occlusal splint · Three-dimensional printing · Subtractive manufacturing · Polyetheretherketone · Cytotoxicity · Cell death · Interleukin-6

Background

Temporomandibular disorders (TMDs) are among the most prevalent musculoskeletal conditions of the orofacial region, with joint-related forms affecting approximately 31% of adults and older adults [1]. Sleep bruxism is frequently reported alongside them, although a causal link remains unclear [2]. Removable intraoral appliances—occlusal splints that provide full coverage of the maxillary or mandibular dental arch—remain a cornerstone of conservative TMD management, redistributing occlusal forces, reducing masticatory muscle hyperactivity, and protecting dental hard tissues from parafunctional wear [3, 4]. Because these appliances are typically worn nocturnally for periods extending from several months to years, their materials remain in prolonged contact with the oral mucosa and saliva. The leaching of residual monomers and additives from these materials, with the potential to induce cytotoxic, apoptotic, or local inflammatory effects, is therefore of considerable clinical relevance [5–8].

Conventional fabrication of occlusal splints relies on two long-established techniques: the manual processing of auto-polymerizing or heat-cured polymethyl methacrylate (PMMA) resins, and the thermoforming of preformed thermoplastic foils—most commonly glycol-modified polyethylene terephthalate (PET-G)—over plaster models [3]. Each presents inherent shortcomings. Auto-polymerizing PMMA undergoes chemical polymerization at room temperature, resulting in a relatively low degree of conversion and elevated residual monomer content compared with industrially polymerized counterparts [9]. The methyl methacrylate and other reactive species that subsequently leach from the polymer matrix have been associated with mucosal irritation, allergic reactions, and in vitro cytotoxicity [10, 11]. Thermoformed PET-G foils, although chemically stable and easy to process, are limited by their thermoplastic nature in terms of long-term intraoral performance [8].

The integration of computer-aided design and computer-aided manufacturing (CAD/CAM) technologies has substantially expanded the range of materials available for occlusal splint fabrication [12, 13]. Subtractive manufacturing involves the milling of industrially polymerized blanks, including conventional cross-linked PMMA-based polymers and, more recently, high-performance thermoplastics such as polyetheretherketone (PEEK), which offers high fracture toughness together with excellent chemical and biological inertness [14–16]. Industrial polymerization under controlled temperature and pressure yields a highly cross-linked structure with low residual monomer content [5, 17]. Additive manufacturing, particularly stereolithography and digital light processing, enables the layer-by-layer photopolymerization of liquid resins into geometrically complex devices [12, 18]. The biological behavior of additively manufactured splints, however, depends critically on adequate post-processing, because incomplete polymerization may leave reactive monomers and photoinitiators that elute into the surrounding environment [17–19]. Although early reports raised concerns about the cytotoxicity of certain printable splint resins [7], more recent evidence indicates that, with optimized washing and post-curing protocols, contemporary printable materials can achieve viability levels comparable to milled and conventional alternatives [18, 20].

Despite the increasing clinical adoption of digital workflows, a direct comparison of the four fabrication routes currently used for occlusal splints under identical experimental conditions, combining cytotoxicity, cell death, and inflammatory endpoints, is still lacking [5, 7, 8, 21]. Flow cytometric analysis with Annexin V/PI staining distinguishes viable from non-viable cells and provides a more sensitive indicator of subclinical cellular injury than viability assays alone [7]. Interleukin-6 (IL-6), a pro-inflammatory cytokine released by oral fibroblasts in response to leached resin components, serves as a sensitive biomarker of low-grade inflammatory stimulation [22, 23]. Surface roughness was additionally evaluated to characterize the specimen surfaces, since surface properties contribute to the overall biological performance of dental materials [24] and values exceeding 0.2 µm have been reported as a threshold for clinically relevant plaque retention [25]. Therefore, the present in vitro study aimed to evaluate the cytotoxicity, cell death, IL-6 release, and surface roughness of five contemporary occlusal

splint materials representing four fabrication techniques: CAD/CAM milling (PMMA and PEEK), 3D printing (photopolymer resin), thermoforming (PET-G), and conventional auto-polymerization (PMMA).

Materials and methods

Five contemporary occlusal splint materials produced by four fabrication techniques—CAD/CAM milling, 3D-printing, thermoforming, and conventional auto-polymerization—were evaluated (Table 1), and the study workflow is summarized in Fig. 1.

Specimen preparation

In accordance with the sample-preparation requirements of ISO 10993-12:2012 [26] and each manufacturer's instructions, 240 disc-shaped specimens (15 mm in diameter, 2 mm in thickness) were prepared, comprising 48 specimens for each of the five materials. Within every material group, specimens were allocated to four subgroups ($n = 12$), corresponding to the cell viability, cell death, interleukin-6 (IL-6) release, and surface roughness assessments. All materials were used in the form supplied by the manufacturer for occlusal splint fabrication. According to the manufacturers' instructions for use, M-PM Disc (clear) is indicated for occlusal splints and drilling templates [27], PEEK BioSolution for occlusal splints, with bruxism and abnormal jaw function among its listed indications [28], Duran for prosthetic and orthodontic splint therapy including bruxism and occlusal splints [29], and Dental LT Clear V2 Resin for occlusal splints and night guards in patients diagnosed with temporomandibular joint disorder and/or bruxism [30]. The auto-polymerizing acrylic resin (Akrodent) is marketed for orthodontic appliance fabrication; although not specifically indicated for occlusal splints, it is used for this purpose in clinical practice and has been evaluated as an occlusal splint material in a previous study [31].

For the milled-PMMA (M-PM Disc, clear; Merz Dental GmbH, Lütjenburg, Germany) and milled-PEEK (PEEK BioSolution, A2/B2; Merz Dental GmbH, Lütjenburg, Germany) groups, disc geometries were designed in exoCAD DentalCAD software (exoCAD GmbH, Darmstadt, Germany) and machined from prefabricated blocks on a milling unit (Ceramill Motion 2; Amann Girrbach AG, Koblach, Austria).

Specimens of the printed-resin group (Dental LT Clear Resin; Formlabs, Somerville, MA, USA) were modeled in TinkerCAD (Autodesk, San Francisco, CA, USA), exported as STL files, and fabricated on a stereolithographic

Table 1 Brand names, material classes, compositions, and manufacturers of the materials evaluated in this study

Material	Material class	Composition	Manufacturer
M-PM Disc, clear	CAD/CAM-milled PMMA (Milled-PMMA)	PMMA and cross-linked polymers based on methacrylic acid esters; Residual peroxide as dibenzoyl peroxide; MMA may be contained as residual monomer up to max. 1%.	Merz Dental GmbH, Lütjenburg, Germany
PEEK BioSolution, A2/B2	CAD/CAM-milled inorganically reinforced PEEK (Milled-PEEK)	PEEK ≈ 80%; TiO_2 ≈ 20%; TiO_2-based pigment < 1%	Merz Dental GmbH, Lütjenburg, Germany
Dental LT Clear Resin	3D-printed (SLA) photopolymer (Printed-Resin)	Bis-DMA (50–70%); UDMA (25–45%); methacrylate monomer(s) (7–10%); photoinitiator(s) (<2%)	Formlabs Inc., Somerville, MA, USA
Duran	Thermoformed thermoplastic (Thermoformed PET-G)	PET-G	Scheu-Dental GmbH, Iserlohn, Germany
Akrodent	Auto-polymerizing PMMA-based acrylic resin (Auto-polymerizing PMMA)	Powder: PMMA (>95%); BPO initiator (<2%); pigments (trace). Liquid: MMA (>90%); EGDMA cross-linker (2–5%); DMPT activator (<1.5%); HQ (<0.1%)	Koca Kimya ve Dental Ltd. Şti. (Akrodent), Ankara, Turkey

Abbreviations: *PMMA* poly(methyl methacrylate), *MMA* methyl methacrylate, *PEEK* polyetheretherketone, *TiO_2* titanium dioxide, *Bis-DMA* bisphenol A dimethacrylate, *UDMA* urethane dimethacrylate, *PET-G* polyethylene terephthalate glycol-modified, *EGDMA* ethylene glycol dimethacrylate, *DMPT N* N-dimethyl-p-toluidine, *HQ* hydroquinone

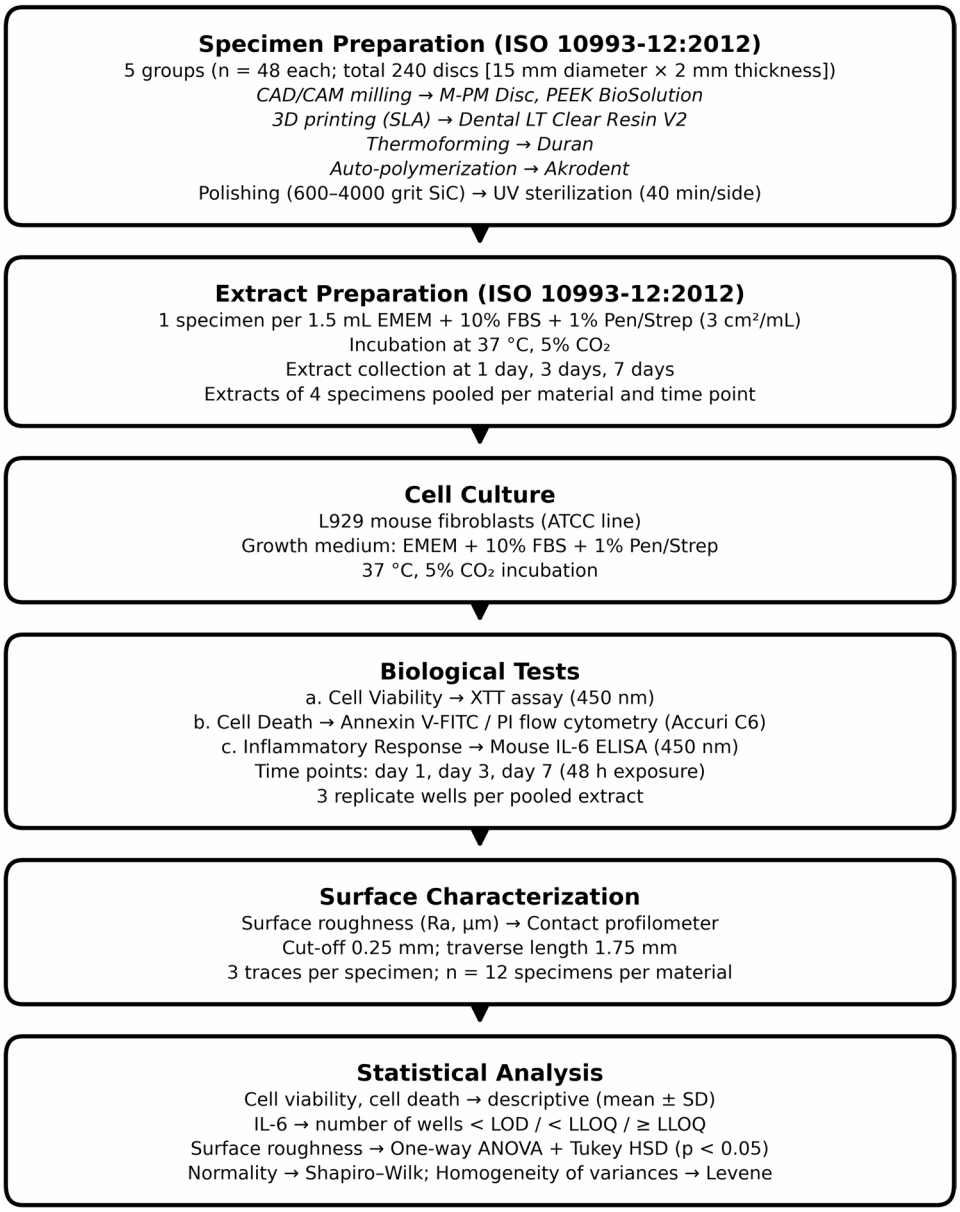


Fig. 1 Flowchart of study design

printer (Form 2; Formlabs, Somerville, MA, USA) at the manufacturer's recommended settings with a 100-µm layer thickness. Printed discs were removed from the build platform and cleaned in an automated unit (Form Wash; Formlabs, Somerville, MA, USA) using 99% isopropyl alcohol for 15 min, followed by a further 5-min immersion in fresh 99% isopropyl alcohol to eliminate residual uncured resin. After air-drying at room temperature for at least 30 min, the specimens were post-cured for 60 min at 60 °C in a UV curing unit (Form Cure; Formlabs, Somerville, MA, USA), as directed by the manufacturer.

For the thermoformed PET-G group, PET-G sheets (Duran, 2.0 mm; Scheu-Dental GmbH, Iserlohn, Germany) were thermally softened in a thermoforming device (Ministar S; Scheu-Dental GmbH, Iserlohn, Germany) and adapted over a stone model carrying standardized disc-shaped impressions according to the manufacturer's instructions, after which the specimens were trimmed to the specified diameter.

The auto-polymerizing PMMA group (Akrodent; Koca Kimya ve Dental, Ankara, Turkey) was prepared by mixing the powder and liquid at the manufacturer's specified ratio; the mixture was packed into a standardized

silicone mold and cured in a pressure pot at 2.0 bar and 55 °C for 10 min according to the manufacturer's instructions.

All specimens were sequentially polished with 600-, 800-, 1000-, 2000-, and 4000-grit silicon carbide papers under continuous water cooling, ultrasonically cleaned in distilled water for 5 min, and air-dried. Before biological testing, each surface was sterilized under ultraviolet light for 40 min per side within a laminar-flow cabinet (Airstream Class II BSC; ESCO, South Yorkshire, UK).

Preparation of material extracts

Material extracts were prepared in accordance with ISO 10993-12:2012 [26]. Each sterilized specimen was transferred to an individual well of a 12-well plate containing 1.5 mL of Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin, corresponding to the surface area-to-volume ratio of 3 cm^2/mL specified in that standard for materials with a thickness > 1.0 mm; wells without specimens served as the control group. At each time point, the extracts obtained from the four specimens were pooled to generate a single pooled extract for each material, which was subsequently analyzed in three technical replicate wells, in accordance with the minimum of three replicates specified in ISO 10993-5:2009 (8.1) [32]. The plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere, and the resulting extracts were harvested after 1, 3, and 7 days, passed through a 0.22 µm polyethersulfone (PES) filter, and stored at − 20 °C until analysis.

Cell culture preparation

The L929 mouse fibroblast line (American Type Culture Collection; Manassas, VA, USA) was cultured in EMEM containing 10% FBS and 1% penicillin–streptomycin and kept at 37 °C in a humidified 5% CO_2 incubator, with medium renewal every three days and passaging after trypsinization.

Cell viability

Cytotoxicity was evaluated with the XTT assay (Cell Proliferation Kit II; Roche Diagnostics GmbH, Mannheim, Germany) [33], which reflects viable-cell metabolism through the mitochondrial reduction of XTT to formazan. L929 cells were plated at 1×10^4 cells/well in 96-well plates and left to adhere for 24 h. The medium was then discarded and replaced with 100 µL of each material extract. After a further 48 h, within the 24–96 h range specified by the manufacturer [33], formazan absorbance was recorded at 450 nm on a microplate spectrophotometer (Thermo Scientific, Vantaa, Finland). Cell viability was calculated for each technical replicate well by dividing its absorbance by the mean absorbance of the untreated control wells from the corresponding time point and multiplying by 100. Thus, the mean untreated control absorbance at each time point served as the 100% normalization reference. Each pooled extract was assayed in three replicate wells, and testing of the eluates followed ISO 10993-5:2009 [32].

Cell death analysis

Cell death was examined using day 1, day 3, and day 7 extracts, with a 48-h exposure at each time point. L929 fibroblasts were seeded in 48-well plates at 7×10^4 cells/well and allowed to attach and stabilize for 24 h under standard conditions (37 °C, 5% CO_2, humidified atmosphere). The medium was subsequently removed and replaced with 200 µL of the corresponding material extract. After 48 h of treatment, the cells were washed with phosphate-buffered saline (PBS), resuspended in 100 µL of binding buffer, and stained with 5 µL of Annexin V-FITC and 5 µL of PI, with the final volume adjusted to 500 µL (Annexin V-FITC/PI; BD Biosciences, San Jose, CA, USA). Annexin V-FITC (FL1) and PI (FL2) fluorescence was acquired simultaneously on a flow cytometer

(Accuri C6; BD Biosciences, San Jose, CA, USA). Total cell death was calculated as the sum of early apoptotic (Annexin V^+/PI^-), late apoptotic/secondary necrotic (Annexin V^+/PI^+), and primary necrotic (Annexin V^-/PI^+) cells. Each pooled extract was assayed in three replicate wells.

Inflammatory response

L929 fibroblasts were seeded in 12-well plates in EMEM supplemented with 10% FBS and 100 U/mL penicillin–streptomycin and allowed to attach for 24 h at 37 °C in a humidified 5% CO_2 atmosphere, after which the medium was replaced with the corresponding material extract. After 48 h of exposure to the day 1, day 3, and day 7 extracts, IL-6 concentrations in the cell culture supernatants were determined using a Mouse IL-6 ELISA Kit (Elabscience Biotechnology, Houston, TX, USA). 100 µL of cell supernatant was used for each group. The absorbance was read at 450 nm using a microplate spectrophotometer. Each pooled extract was assayed in three replicate wells. The assay had a manufacturer-reported sensitivity (LOD) of 18.75 pg/mL and a quantitative range of 31.25–2000 pg/mL, with the lowest calibrator (31.25 pg/mL) defining the lower limit of quantification (LLOQ) [34]. IL-6 results were reported as the number of replicate wells below the LOD, detectable but below the LLOQ, and quantifiable (≥ LLOQ), with individual concentrations given for quantifiable wells; group means were not calculated because not all replicate wells were quantifiable.

Surface roughness measurement

Surface roughness was measured with a contact profilometer (Surtronic 25; Taylor Hobson, Leicester, UK) calibrated against a reference standard before use. For each specimen, three random linear traces were taken from the center using a 1.75 mm traverse length, a 0.25 mm cut-off, and a 0.5 mm/s scanning speed; the mean Ra (µm) was calculated from the three readings.

Statistical analysis

For each material and time point, a single pooled extract was prepared; thus, the three replicate wells served as technical replicates of one extract preparation rather than independent extractions. As the biological data lack independent replication at the material-extraction level, inferential statistical analysis was not conducted for these endpoints. Cell viability and cell death results are reported descriptively as mean ± standard deviation of the three replicate wells, and IL-6 results as the number of replicate wells in each detection category, with no p-values, effect sizes, or significance groupings provided. No a priori sample-size calculation was performed. Surface roughness was measured on independent specimens ($n = 12$ per material). Statistical analyses of these data were performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Normality was evaluated using the Shapiro–Wilk test and homogeneity of variance with Levene's test. Data were analyzed by one-way ANOVA followed by Tukey's HSD post hoc test, with significance defined as $p < 0.05$.

Results

Cytotoxic effects of occlusal splint materials

Cell viability of the tested materials across the different time intervals is presented in Fig. 2; Table 2. Cell viability was expressed relative to the mean absorbance of the untreated control wells at each time point, which served as the 100% normalization reference; the control is therefore not shown as a separate group in Fig. 2. As only one pooled extract was available per material and time point, the results are reported descriptively.

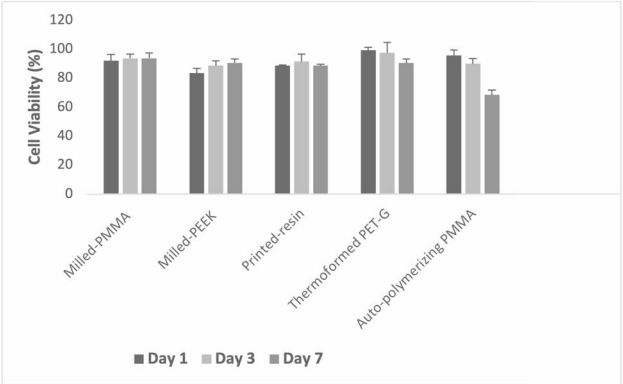


Fig. 2 Cell viability of L929 fibroblast cells exposed to occlusal splint material extracts. Bars represent mean ± SD of three replicate wells from a single pooled extract per material and time point. Cell viability was calculated relative to the mean absorbance of the untreated control wells at the corresponding time point, which served as the 100% normalization reference and is therefore not shown as a separate bar. As the replicate wells are technical replicates, the data are presented descriptively, and no statistical comparisons were performed

Table 2 Cell viability (%) of L929 fibroblast cells exposed to days 1, 3, and 7 medium extracts of occlusal splint materials

Material	Day 1	Day 3	Day 7
Milled-PMMA	92.06 ± 4.24	93.76 ± 3.02	93.78 ± 3.78
Milled-PEEK	83.52 ± 3.40	88.73 ± 3.39	90.60 ± 2.73
Printed-Resin	88.72 ± 0.54	91.49 ± 5.17	88.69 ± 0.91
Thermoformed PET-G	99.38 ± 1.86	97.51 ± 7.10	90.57 ± 2.72
Auto-polymerizing PMMA	95.71 ± 3.74	89.93 ± 3.71	68.44 ± 3.48

Values are presented as mean ± standard deviation of three replicate wells from a single pooled extract per material and time point. As the replicate wells are technical replicates, no inferential statistical analysis was performed. Cell viability was calculated relative to the mean absorbance of the untreated control wells at the corresponding time point, which served as the 100% normalization reference

On day 1, the thermoformed PET-G group showed the highest cell viability (99.38 ± 1.86%), followed by auto-polymerizing PMMA (95.71 ± 3.74%), milled-PMMA (92.06 ± 4.24%), printed-resin (88.72 ± 0.54%), and milled-PEEK (83.52 ± 3.40%).

On day 3, values ranged from 88.73 ± 3.39% for milled-PEEK to 97.51 ± 7.10% for thermoformed PET-G, with all materials remaining above the 70% threshold defined in ISO 10993-5 [32].

On day 7, the auto-polymerizing PMMA group showed the lowest cell viability (68.44 ± 3.48%) and was the only material to fall below this threshold. The remaining groups ranged from 88.69 ± 0.91% for printed-resin to 93.78 ± 3.78% for milled-PMMA.

Across the evaluated intervals, cell viability remained stable in the milled-PMMA, milled-PEEK, printed-resin and thermoformed PET-G groups, whereas the auto-polymerizing PMMA group showed a progressive decrease from 95.71 ± 3.74% on day 1 to 89.93 ± 3.71% on day 3 and 68.44 ± 3.48% on day 7.

Cell death analysis

The total cell death results for the tested materials at different time intervals are presented in Fig. 3; Table 3. Representative flow cytometric dot plots are shown in Fig. 4. As only one pooled extract was available per material and time point, the results are reported descriptively. An untreated control was analyzed in parallel in three technical replicate wells (1.50 ± 0.30%). Because these wells represented technical replicates of the same control condition rather than independent biological replicates, the control value is reported descriptively and the same reference value is displayed at each time point in Fig. 3.

On day 1, total cell death ranged from 1.40 ± 0.75% for milled-PEEK to 2.66 ± 0.77% for printed-resin, with intermediate values for milled-PMMA (1.90 ± 0.75%), thermoformed PET-G (1.57 ± 0.60%) and auto-polymerizing PMMA (1.42 ± 0.48%).

On day 3, values ranged from 1.53 ± 0.21% for milled-PEEK to 2.52 ± 1.02% for auto-polymerizing PMMA.

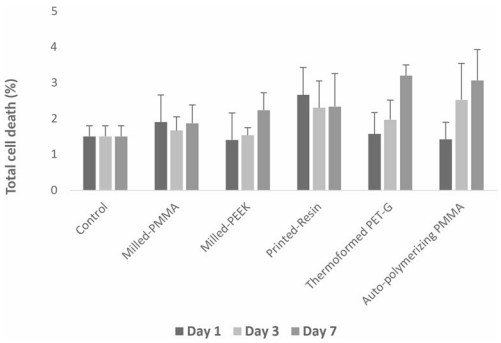


Fig. 3 Total cell death of L929 fibroblast cells exposed to occlusal splint material extracts. Bars represent mean ± SD of three replicate wells from a single pooled extract per material and time point. An untreated control was analyzed in three technical replicate wells, and the same reference value is shown at each time point. As the replicate wells are technical replicates, the data are presented descriptively, and no statistical comparisons were performed

Table 3 Total cell death (%) of L929 fibroblast cells exposed to days 1, 3, and 7 medium extracts of occlusal splint materials

Material	Day 1	Day 3	Day 7
Control	1.50 ± 0.30	1.50 ± 0.30	1.50 ± 0.30
Milled-PMMA	1.90 ± 0.75	1.67 ± 0.38	1.87 ± 0.51
Milled-PEEK	1.40 ± 0.75	1.53 ± 0.21	2.23 ± 0.49
Printed-Resin	2.66 ± 0.77	2.30 ± 0.75	2.33 ± 0.93
Thermoformed PET-G	1.57 ± 0.60	1.97 ± 0.55	3.20 ± 0.30
Auto-polymerizing PMMA	1.42 ± 0.48	2.52 ± 1.02	3.07 ± 0.86

Values are presented as mean ± standard deviation of three replicate wells from a single pooled extract per material and time point. As the replicate wells are technical replicates, no inferential statistical analysis was performed. An untreated control was analyzed in three technical replicate wells (1.50 ± 0.30%), and the same reference value is given at each time point

On day 7, the highest values were recorded for thermoformed PET-G (3.20 ± 0.30%) and auto-polymerizing PMMA (3.07 ± 0.86%), followed by printed-resin (2.33 ± 0.93%), milled-PEEK (2.23 ± 0.49%) and milled-PMMA (1.87 ± 0.51%).

Across the evaluated intervals, total cell death remained stable in the milled-PMMA (1.90 ± 0.75% to 1.87 ± 0.51%) and printed-resin (2.66 ± 0.77% to 2.33 ± 0.93%) groups, whereas the milled-PEEK, thermoformed PET-G and auto-polymerizing PMMA groups showed increases from day 1 to day 7, most markedly in the thermoformed PET-G (1.57 ± 0.60% to 3.20 ± 0.30%) and auto-polymerizing PMMA (1.42 ± 0.48% to 3.07 ± 0.86%) groups.

Evaluation of inflammatory response

IL-6 levels following exposure to the material extracts are presented in Table 4. As only one pooled extract was available per material and time point, the results are reported descriptively as the number of replicate wells in each detection category. An untreated control was analyzed in parallel on the same plate in three technical replicate wells, all of which were below the LOD.

IL-6 was below the LOD in all milled-PEEK wells and in all day-7 wells except those of the auto-polymerizing PMMA group. A detectable signal below the LLOQ was observed on day 1 in two of three wells for milled-PMMA and thermoformed PET-G and in one of three wells for printed-resin and auto-polymerizing PMMA, and on day 3 in two of three wells for thermoformed PET-G. Only the auto-polymerizing PMMA group on day 7 yielded quantifiable IL-6, in two of three wells (33.78 and 42.55 pg/mL); the third well was detectable but below the LLOQ. All remaining wells were below the LOD.

Surface roughness measurements

Mean Ra values of the tested materials are given in Table 5. One-way ANOVA revealed a statistically significant difference among the tested material groups ($p = 0.005$). The milled-PMMA group exhibited the highest Ra value

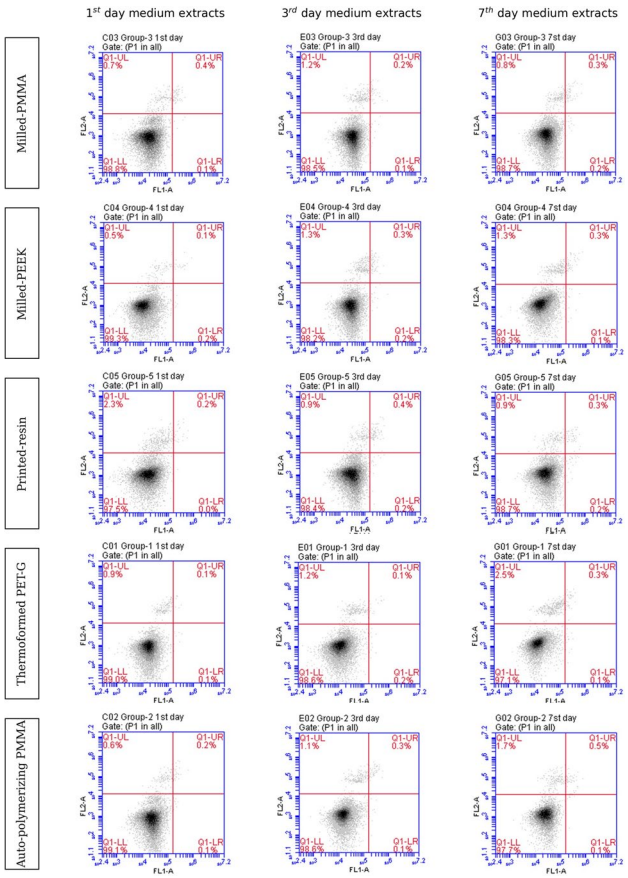


Fig. 4 Annexin V/PI staining of indicated cell groups. Cells were treated with the day 1, day 3, and day 7 medium extracts of occlusal splint materials for 48 h and then subjected to flow cytometric analysis (LL: viable cells; LR: early apoptotic cells; UR: late apoptotic/secondary necrotic cells; UL: primary necrotic cells)

Table 4 IL-6 release from L929 fibroblast cells exposed to days 1, 3, and 7 medium extracts of occlusal splint materials

Material	Day 1	Day 3	Day 7
Control	< LOD (3 wells)	< LOD (3 wells)	< LOD (3 wells)
Milled-PMMA	< LOD (1 well) < LLOQ (2 wells)	< LOD (3 wells)	< LOD (3 wells)
Milled-PEEK	< LOD (3 wells)	< LOD (3 wells)	< LOD (3 wells)
Printed-Resin	< LOD (2 wells) < LLOQ (1 well)	< LOD (3 wells)	< LOD (3 wells)
Thermoformed PET-G	< LOD (1 well) < LLOQ (2 wells)	< LOD (1 well) < LLOQ (2 wells)	< LOD (3 wells)
Auto-polymerizing PMMA	< LOD (2 wells) < LLOQ (1 well)	< LOD (3 wells)	< LLOQ (1 well) quantifiable (2 wells): 33.78 and 42.55 pg/mL

Values indicate the number of replicate wells (of three) in each category: below the limit of detection (< LOD, 18.75 pg/mL), detectable but below the lower limit of quantification (< LLOQ, 31.25 pg/mL), or quantifiable (≥ LLOQ). Group means are not reported because not all replicate wells were quantifiable

Table 5 Mean and standard deviation of surface roughness (Ra ± SD) values of occlusal splint materials

Material	Mean Ra ± SD (µm)
Milled-PMMA	0.25 ± 0.07^{a}
Milled-PEEK	0.15 ± 0.04^{b}
Printed-Resin	0.22 ± 0.04^{ab}
Thermoformed PET-G	0.15 ± 0.04^{b}
Auto-polymerizing PMMA	0.19 ± 0.10^{ab}

One-way ANOVA showed a significant difference among materials ($p = 0.005$). Different superscript lowercase letters indicate statistically significant differences among groups according to Tukey's test ($p < 0.05$)

(0.25 ± 0.07 µm), differing significantly from the milled-PEEK group (0.15 ± 0.04 µm) and the thermoformed PET-G group (0.15 ± 0.04 µm) ($p < 0.05$). The printed-resin group (0.22 ± 0.04 µm) and the auto-polymerizing PMMA group (0.19 ± 0.10 µm) showed intermediate values that did not differ significantly from any other group ($p > 0.05$).

Discussion

The increasing clinical use of digitally manufactured occlusal splint materials has made the biocompatibility of these appliances, which remain in prolonged intraoral contact during nightly wear, clinically important to evaluate. While the biological properties of conventional splint resins are well-documented, data on 3D-printed and high-performance polymer alternatives remain limited [5, 6, 8, 17]. This in vitro study evaluated cell viability, cell death, IL-6 release, and surface roughness of five occlusal splint materials produced using four distinct manufacturing techniques over a seven-day evaluation period. The milled and printed groups showed favorable in vitro biological responses, with cell viability consistently exceeding the 70% threshold defined in ISO 10993-5 at all-time points. In contrast, the auto-polymerizing PMMA group showed a progressive decline in viability and was the only group with quantifiable IL-6, while the thermoformed PET-G group showed the highest total cell death value on day 7. Surface roughness also differed significantly among the materials.

Mitochondrial-activity assays alone cannot reliably distinguish growth inhibition from cell death [6]; cytotoxicity (XTT) was therefore complemented with cell death and IL-6 endpoints. The biological differences observed among the materials may be related to differences in their chemistry and processing, although these variables were not manipulated independently in the present design and their individual contributions cannot be established. The methacrylate-based materials share the same principal monomer (methyl methacrylate, MMA) but differ in polymerization: auto-polymerizing PMMA is processed at room temperature with a chemical initiator–accelerator system and retains a larger proportion of unreacted monomer than heat-polymerized resins [9], whereas industrially polymerized (milled) PMMA is produced under elevated temperature and pressure, achieving a high degree of conversion with minimal residual monomer [17]. Residual methacrylate monomers have been reported to enhance the formation of reactive oxygen species and to induce apoptosis in cultured cells [35, 36]; methacrylate–glutathione adduct formation has also been demonstrated, although a direct correlation between glutathione depletion and cytotoxicity could not be established [37]. As leachable species were not chemically characterized in the present study, these pathways can only be regarded as plausible explanations for the observed responses rather than mechanisms demonstrated here. Accordingly, Wedekind et al. [17] quantified residual monomer release from 3D-printed, milled and conventional splint materials by gas chromatography/mass spectrometry and reported markedly lower MMA elution from milled PMMA blanks than from conventional powder/liquid materials in methanol extracts, whereas no methacrylates were detectable in the corresponding aqueous extracts. In line with this, Kollmuss et al. [21] found that industrially polymerized milled PMMA was among the materials eliciting the weakest cytotoxic response in gingival fibroblasts, consistent with the higher degree of conversion achieved under industrial polymerization conditions. Wei et al. reached a comparable conclusion using

high-performance liquid chromatography, detecting residual MMA only in a compression-moulded PMMA and not in the CAD/CAM-processed polymers, and reported greater cell proliferation and no apoptosis in gingival fibroblasts exposed to the CAD/CAM materials [38]. By contrast, PEEK contains no methacrylate monomers and is machined from fully polymerized stock; it is generally regarded as chemically stable and bioinert [14–16], while thermoformed PET-G is extruded from highly polymerized sheets but may release low-molecular-weight constituents after thermal processing.

Apoptosis is a regulated form of programmed cell death that is morphologically and biochemically distinct from necrosis [39]. In the present study, the milled-PMMA, milled-PEEK, and printed-resin groups maintained favorable biological profiles, with stable viability and low total cell death throughout the evaluation period. The milled-PEEK group showed the lowest viability among the tested materials on day 1 (83.52 ± 3.40%), although this value remained well above the 70% threshold defined in ISO 10993-5 [32]. Viability increased to above 90% by day 7, indicating that the early reduction was transient and of no cytotoxic relevance. This is consistent with the recognized chemical and biological inertness of PEEK [40]. In contrast, the auto-polymerizing PMMA group exhibited the most pronounced response, with a progressive decline in viability to 68.44% by day 7 and quantifiable IL-6 in two of three wells at the same time point. This was the only material to fall below the 70% viability limit defined in ISO 10993-5 [32]. The auto-polymerizing resin tested here is marketed for orthodontic appliance fabrication rather than specifically for occlusal splints, and its biological behavior in this application has not previously been characterized; the present findings are therefore of direct clinical relevance when such materials are used for splint fabrication. These findings are broadly consistent with previous reports. Bürgers et al. [5] evaluated Dental LT Clear, a milled PMMA from the same manufacturer, and other splint resins on L929 and gingival fibroblasts using a direct-contact model, in which cells were seeded onto the specimens so that both surface characteristics and eluted species contributed to the response, and found no significant differences between fabrication routes when materials were grouped by manufacturing method. In gingival fibroblasts, the difference between the two milled resins was highly significant, indicating that variation within a fabrication route may exceed variation between routes. Similarly, Haugli et al. [6] reported that the cytotoxicity of an auto-polymerized splint material was largely eliminated when specimens were pre-stored in distilled water. However, in that study the milled specimens also caused a significant reduction in viability, a result not observed in the present study; this may be related to the use of a human oral epithelial cell line rather than L929 fibroblasts, and to extraction under agitation rather than static conditions. Kollmuss et al. [21] similarly detected no apoptotic activity in gingival fibroblasts or oral keratinocytes exposed to eluates of printed and milled splint materials, which is in line with the low total cell death observed across all groups in the present study, despite the different cell models, material sets, and cell death assays (Annexin V/PI staining after 48 h versus caspase-3/7 activity after 5 h). The favorable profile of the printed-resin group may be related to its standardized post-processing, which has been shown to reduce residual monomer and photoinitiator content; insufficiently post-processed printed resins, by contrast, can exhibit considerable cytotoxicity [19]. Surface finishing appears to be equally relevant: Bieger et al. reported that human gingival fibroblasts cultivated directly on unpolished specimens of the same printed resin used here showed essentially no viability, whereas polishing restored cell behavior to a level comparable with conventionally processed and milled materials [41]. In the present study all specimens were polished before testing, which may in part account for the favorable profile observed for this material. Weżgowiec et al. [8] evaluated the same printed resin (Dental LT Clear) and the same thermoplastic (Duran) using human gingival fibroblasts and reported that extracts of Dental LT Clear reduced metabolic activity. In contrast, Duran extracts did not, although in both cases the reduction remained below the 30% cytotoxicity threshold of ISO 10993-5 [32]. In the present study, both materials likewise maintained viability well above this threshold.

The inflammatory response, assessed through IL-6 release, was limited in most conditions. IL-6 remained below the limit of detection of the assay in all milled-PEEK wells and in most remaining wells; quantifiable IL-6 was obtained only in two of three wells of the auto-polymerizing PMMA group on day 7. This coincided with the lowest cell viability recorded in the study, suggesting that the two endpoints identified the same material as the most reactive. Extracts of two of three auto-polymerizing acrylic resins have been shown to upregulate IL-6

transcription in human gingival fibroblasts, in a manner dependent on the duration of polymerization [23], which offers a possible explanation for the concurrent cytotoxic and inflammatory responses observed for the auto-polymerizing PMMA group. In the only comparable study to have assessed inflammatory response in oral appliance materials, Kollmuss et al. [21] found that IL-6 secretion by gingival fibroblasts was significantly increased by only one of eight tested materials, and that keratinocyte IL-6 levels were unaffected. Our findings are consistent with this, in that a measurable IL-6 response was confined to a single material. The thermoformed PET-G group maintained viability above 90% at all time points, yet showed the highest total cell death value on day 7 ($3.20 \pm 0.30\%$), indicating that the two endpoints did not always identify the same materials.

Surface roughness differed significantly among the materials, with the milled-PMMA group exhibiting the highest Ra value and the milled-PEEK and thermoformed PET-G groups the lowest. These values lie close to the 0.2 µm threshold proposed by Bollen et al. [25], below which further reductions in roughness do not decrease bacterial adhesion; the low Ra of PEEK is consistent with Benli et al. [42]. Wężgowiec et al. [8] evaluated the same printed resin and thermoplastic and found no significant differences in Ra between materials after polishing, whereas significant differences persisted in the present study, possibly reflecting differences in the finishing protocols applied. Importantly, surface roughness was assessed solely to characterize the specimens; the biological responses were obtained from material extracts and were therefore independent of surface texture.

To our knowledge, this is the first study to evaluate cell viability, cell death and inflammatory response concurrently in the same extracts across four fabrication routes for occlusal splints, including a milled PEEK material that has not previously been examined in this context. Assessing these endpoints in parallel allowed a material to be characterized as biologically acceptable on one endpoint while showing a measurable response on another, as observed for the thermoformed PET-G group, which maintained viability above 90% but showed the highest total cell death value on day 7. Collecting extracts at three separate intervals rather than as a single cumulative eluate further allowed the time course of the biological response to be resolved, which proved relevant for the auto-polymerizing PMMA group, where viability remained above the ISO 10993-5 threshold until day 7. Clinically, these observations indicate that a material may appear acceptable at an early time point yet show a measurable response with prolonged exposure, which is relevant for appliances worn nightly over extended periods.

Several limitations should be acknowledged. First, the experiments were conducted under in vitro conditions and cannot reproduce the complexity of the intraoral environment, including salivary flow, mechanical loading, and the resident microbiome; the findings should therefore be regarded as biological screening data requiring in vivo and clinical confirmation [43]. Second, at each time point the extracts of the four specimens were pooled prior to cell exposure, and the resulting pooled extract was analyzed in three replicate wells. The replicate wells therefore represent technical rather than independent biological replicates and do not capture specimen-to-specimen variability. Only one independently prepared extract was therefore available per material and time point, which does not provide independent replication of the material-extraction process; the biological findings are consequently descriptive and preliminary and require confirmation in studies using independent extraction batches. Third, residual monomer elution was not quantified analytically; without gas chromatography–mass spectrometry, the observed responses could not be linked to specific eluted species. Fourth, only one printed-resin material and one post-processing protocol were evaluated, and the findings should not be generalized to all 3D-printed splint materials. Fifth, L929 mouse fibroblasts, while recommended by ISO 10993-5 [32], do not fully recapitulate the human oral mucosa; the choice of cell model has been shown to substantially influence measured monomer toxicity [44], and confirmatory studies in primary human gingival fibroblasts or three-dimensional oral mucosa models would strengthen extrapolation. Finally, the seven-day period is substantially shorter than the months to years of cumulative nightly wear typical of clinical splint use, warranting longer-term in vivo investigation.

Conclusions

Within the limits of this in vitro study, the five occlusal splint materials displayed distinct biological profiles. The milled-PMMA, milled-PEEK, and printed-resin groups showed favorable in vitro biological responses, with stable viability, low total cell death, and IL-6 below the limit of detection of the assay in most wells. The thermoformed PET-G group maintained viability above 90% but showed the highest total cell death value on day 7. In contrast, the auto-polymerizing PMMA group elicited the most pronounced adverse response, as it was the only material to fall below the 70% viability limit defined in ISO 10993-5 and the only one with quantifiable IL-6, detected in two of three wells on day 7. Surface roughness also differed among the materials, with the milled-PEEK and thermoformed PET-G groups showing the lowest values; as the biological responses were obtained from material extracts, these differences were independent of the observed cellular responses. As only single representative products and one post-processing protocol were evaluated, these findings should not be generalized to milled or 3D-printed splint materials as a class; splint materials should be selected carefully for clinical application, since individual biocompatibility may vary considerably within the same fabrication route. These biological findings are descriptive and require confirmation in studies with independent extraction batches.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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