Development of Bis-GMA-free biopolymer to avoid estrogenicity

Abstract

Objective. Although bisphenol A-glycidyl methacrylate (Bis-GMA)-based dental materials are widely used in dentistry, Estrogenicity from released bisphenol A remains a concern due to possibility of adversely affecting the growth of children and homeostasis of adults. Here, a new family of isosorbide-derived biomonomers were synthesized and experimentally utilized as a matrix of dental sealants to provide physico-mechanical and biological properties comparable to those of a conventional Bis-GMA-based material but without the the potential estrogenicity.

Methods. After synthesis of isosorbide-derived biomonomers (ISDB) by light polymerization, an experimental dental sealant with different silica filler concentrations (0~15 wt%) was characterized and compared to a commercially available Bis-GMA-based sealant. Cytotoxicity and estrogenicity assays were conducted with human oral keratinocytes and estrogen-sensitive MCF-7 cells, respectively.

Results. ISDB-based dental sealants exhibited typical initially smooth surfaces with depth of cure, Vickers hardness, compressive strength/modulus, water resorption/solubility, and flowability comparable to those of the commercial sealant and met the ISO standard for dental sealants and polymer-based restorative materials. Indirect cytotoxicity tests using an extract showed comparable viability among experimental ISDB-based materials and a commercial Bis-GMA-incorporated control. DNA synthesis in MCF-7 cells (a marker of estrogenicity) and the release of bisphenol A under enzymatic incubation were not detected in ISDB-based materials.

Significance. In conclusion, the comparable physico-mechanical properties of ISDB-based materials with their cytocompatibility and lack of estrogenicity suggest the potential

usefulness of ISDBs as a newly developed and safe biomaterial.

Keywords: isosorbide-derived biomonomer; experimental biopolymer; estrogenicity; cytocompatibility; bisphenol free

Introduction

Estrogenicity is caused by a number of synthetic compounds that mimic the physiological activity of estrogen and adversely affects the development of children and hormone homeostasis in adults[1-3]. One out of many synthetic compounds that induce estrogenicity is bisphenol A (BPA), a molecule known to be a core component of Bis-GMA (BPA glycidylmethacrylate), which is the basis of dental restorative composites[4,5]. BPA mimics the estrogen hormone; this is the reason why it is considered as an endocrine disruptor [6]. BPA is potentially released from Bis-GMA-based dental materials, mainly due to incomplete photopolymerization or impurity (i.e. BPA or BIS-GMA) inclusion, which are hydrolytically or biologically degraded into BPA [7]. Thus, BPA and/or its derivatives are possibly released into the oral cavity and might induce unexpected estrogenic effects[8,9], even though BPA is released from Bis-GMA at a low level under in vitro conditions[10]. Overall, health concerns with respect to Bis-GMA and its related products remain, since they contain the BPA moiety within their chemical structure, which cannot be ruled out that emergence of estrogenic BPA occur [9].

Many trials have been performed to replace Bis-GMA monomers in dentistry by 2,2,4,4-tetramethyl-1,3-cyclobutanediol, urethane dimethacrylate, bile acids, and isosorbide itself to reduce possible adverse health issues from BPA[11], but optimal Bis-GMA-free composite systems have not been successfully explored majorly due to lack of physico-mechanical properties. Isosorbide based checmial compounds have been highlighted as possible replacement or supplemented biomonomer in biopolymer complexes due to its safe origin (bioderived from starch glucose), high mechanical strength, biodegradability and biocompatibility, classified by Food and Drug Administration of the United States Government as ‘generally recognized as safe’ [12-17]. Although isosorbides have a bicyclic

chemical structure, which is similar to BPA, and are susceptible to hydrolysis or enzymatic degradation as biodegradable materials, they and their derivatives are considered as safe materials due to their natural origin and eco-friendly characteristics, in contrast to the petrochemically derived Bis-GMA [12,18,19]. However, to the best of our knowledge, the investigation about the estrogenicity, after optimal fabrication of isosorbide-based materials comparable to commercially available medical products in terms of mechanical and biological properties, has not been investigated.

Thus, the aims of this study are the development of isosorbide based monomers, the compositional optimization of them, for application in dental sealant as an examplar medical product with physico-mechanical properties similar to those of Bis-GMA-based materials, and the investigation of estrogenicity from them under enzymatic degradation. Initially, a new synthesis of light polymerizable isosorbide-derived biomonomers (ISDBs) was performed, and the possible byproducts resulting from ISDB-based dental sealant degradation were investigated under enzymatically accelerated hydrolysis to confirm the absence of BPA-related byproducts, which would cause estrogenicity. Furthermore, the physico-mechanical properties and in vitro cytocompatibility/estrogenicity of these materials were determined with a commercial Bis-GMA-based counterpart. The major null hypothesis of this investigationis is that there is difference in myriad properties such as physico-mechanical properties and in vitro cytocompatibility/estrogenicity between commercial Bis-GMA based dental sealant and developed ISDB-based dental sealant.

Materials and Methods

Materials

1,4:3,6-Dianhydro-D-sorbitol (isosorbide, 98%), ethylene carbonate (99%), triethylene glycol dimethacrylate (TEGDMA, 95%), dibutyltin dilaurate (DBTDL, 95%), 2-isocyanatoethyl methacrylate (IEM, 98%), camphorquinone (CQ, 97%), and ethyl 4-dimethylaminobenzoate (EDMAB, 99%) were obtained from Sigma-Aldrich (St Louis, MO, USA) and distilled to remove inhibitors and increase purity. Potassium carbonate (99.5%), ethyl acetate (99.5%), *n*-hexane (99.5%), methanol (99.5%), and chloroform (99.5%) were supplied by Daejung Chem. Co. Ltd. (Seoul, Korea).

^{1}H nuclear magnetic resonance (NMR) spectra were recorded with an AVANCE III HD 850 spectrometer (850 MHz, Bruker, Germany) and $CDCl_3$ as a solvent. All data are given in terms of chemical shift (δ, ppm) downfield from tetramethylsilane. High-resolution mass spectra were recorded using a JMS-700 spectrometer (JEOL, JAPAN) in positive ionization mode. Elemental analysis was performed using a Flash 2000 (Thermo Fisher Scientific, Waltham, MA, USA) elemental analyser.

Fabrication of isosorbide-derived biomonomer

Details of the synthesis are given in the appendix, which are in-house fabricated for the first time. Briefly, isosorbide-derived biomonomer was synthesized into an intermediate chemical compound (bis(2-hydroxyethyl) isosorbide, BHIS) and then to a final monomer (ISDB). The ISDB isosorbide was synthesized according to the manufacturer's procedures (Sigma-Aldrich). Briefly, isosorbide sequentially reacted with ethylene carbonate, potassium carbonate, and IEM to make isosorbide-derived biomonomers. **Fig. 1A** presents the sequential synthesis of BHIS and the ISDB, which was finally prepared by a urethane

coupling reaction between the hydroxyl group of BHIS and the isocyanate group of IEM, making ethylene glycol linkages. The isosorbide core acts as a rigid segment, and the ethylene glycol and urethane groups on both sides of the isosorbide core were added for elasticity (less brittle) and to reinforce the mechanical properties by hydrogen bonding, potentially giving more toughness. Both termini of ISDB have polymerizable methacrylate groups to crosslink other methacrylates. Hydrolysis of ISDB ester bonds under esterase generated the degradation product of isosorbide-ethylene glycol, not bisphenol A, meaning less concerns about estrogenicity from estrogen mimicking structure like Bisphenol A (Fig. 1B). The yield of BHIS and ISDB synthesis was 67% and 91% respectively. Detail methodology of synthesis was given in supplementary file.

* ISDB: (((3R,3aR,6S,6aR)-hexahydrofuro[3,2-b]furan-3,6-diyl)bis(oxy))bis(ethane-2,1-diyl) bis((4-methyl-3-oxopent-4-en-1-yl)carbamate)

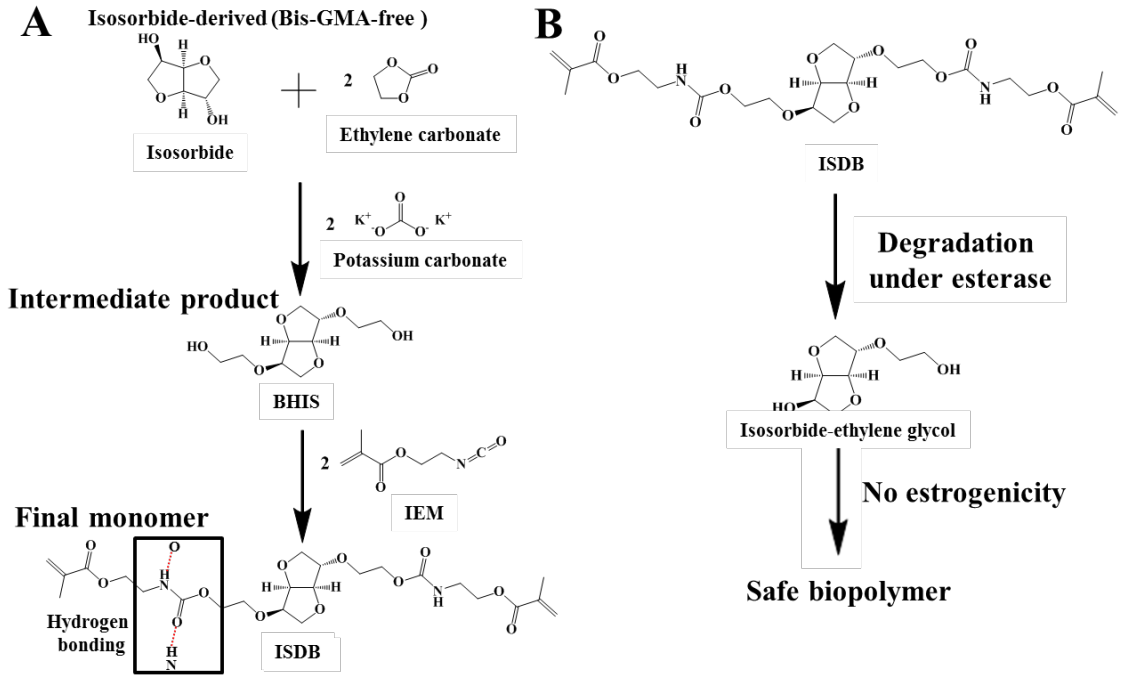
Measurement of viscosity

The viscosity of the ISBD was measured by means of a viscometer (DV 2T, Brookfield, Massachusetts, USA) and compared with other monomers: Bis-GMA and TEGDMA. For the viscosity measurements, the samples were placed directly on the plate and the measurement was carried out in a dark room at 25 ˚C at a shear rate range of 0 to 50 rpm.

Fabrication of dental sealant

Commercially available dental sealant (Concise, 3M) consists of 2 kinds of matrix resin monomers (Bis-GMA and TEGDMA) and was chosen as the control material. The synthesized ISDB was used instead of Bis-GMA. The synthesized ISDB was mixed with TEGDMA as an inert diluent and CQ and EDMAB as photoinitiators. The ratio among ISDB,

TEGDMA, CQ, and EDMAB was 29.5:69.5:0.5:0.5 (wt%). Silanated silica microparticles (Polysciences, Warrington, PA, USA) were added in quantities of 1, 5, 10 or 15 wt% relative to the total amount of experimental dental sealant, partially replacing the ISDB to optimize the physico-mechanical properties comparable to commercial one (Fig. 1C). Amounts of filler were determined based on filler contents from other sealant materials. 4-(dimethylamino)-benzene ethanol was used for photoinitiator for the commercial dental sealant. An LED curing light gun (Litex 695, Dentamerica Industry, 1000±56 mW/cm_2) was used for polymerization. Example of application with experimental and commercial dental sealant applied to tooth groove was shown (Fig. 1D). Details are given in the appendix.



C

Composition of experimental groups (wt%)						
Group	0%	1%	5%	10%	15%	CS
Monomner (ISDB)	99	98	94	89	84	89.5~94.5*
Photo initiators	1	1	1	1	1	0.5
Silane treated silica	0	1	5	10	15	5~10

* Triethylene glycol dimethacrylate and BIS-GMA

D

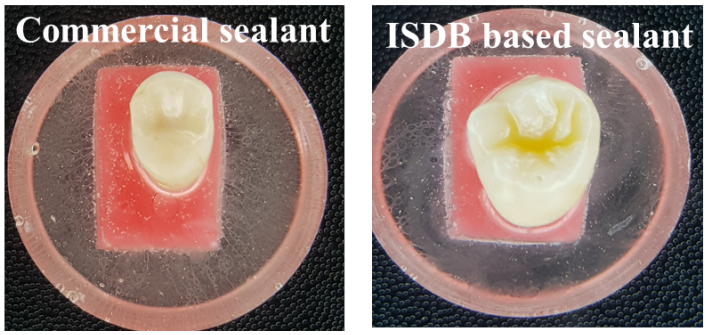


Figure 1. Synthesis of isosorbide-derived biomonomers (ISDBs) for dental restorative materials and experiments in this study. (A) Structure of the starting monomer, intermediate product, and final monomer. (B) Hydrolysis of ISDB ester bonds under esterase generated the degradation product of isosorbide-ethylene glycol, not bisphenol A. (C) Composition of experimental groups. (D) Application of Bis-GMA based commercially available or ISDBs based Bis-GMA-free biopolymer to tooth groove as dental sealant.

Degradation products from dental sealant

Photopolymerized ISDB based dental sealant (h = 1 mm and ϕ = 15 mm for 40 s on each side) were aged in deionized water (DW, 3 cm^2/mL) for 2 weeks at 37 °C with 10 mg/mL porcine

esterases (Sigma, >15 units/mg) to mimic biological hydrolysis by enzymes in saliva. The degradation products that were in the water were extracted into ethyl acetate and washed with distilled water three times. The organic layer was dried and evaporated in a vacuum oven at 60 °C for 24 hr to remove ethyl acetate. The dry extract was mixed with methylene chloride and analysed by a gas chromatograph mass spectrometer (GC/MS, TSQ 8000 Evo, Thermo Fisher Scientific) system.

Physciomechanical characteristics of dental sealant

For evaluating experimental dental sealant compared to commercial product, physicochemical characteristics such as depth of cure, water resorption andsolubility, and compressive strength were investigated. First, the depth of cure and water resorption and solubility (n=5) were investigated according to ISO standard 6874 (depth of cure) and 4049 (water resorption and solubility) [20,21]. For depth of cure, after 40 s of light-curing the top of dental sealant in a stainless steel mould (h = 6 mm and ϕ = 4 mm) and removing the uncured material with the plastic spatula, the height of the polymerized materials was measured with a micrometer, and half of the measured height was determined as the depth of cure. A water resorption and solubility test was performed with a polymerized specimen (h = 1 mm and ϕ = 15 mm). Briefly, after mold was filled with dental sealant, light-curing was performed by overlapping irradiation method (5 times x 40 s per each exposure). After 24 hr incubation in desicators at 37 °C, dry weight was measured as m1 (mg). Then, specimens were immersed in water for 7 d at 37 °C. When the removed specimen was dried until free from visible moisture, weight was measured as m2 (mg). Finally, after fully drying of specimen under desiccators to have costant mass, m3 (mg) was detected. Water resorption was determined by (m2-m3)/V (V is the volume of the specimen, mm^3). Water solubility was

calculated by (m1-m3)/V.

Next, for compressive testing, specimens (n=10) were produced using a stainless steel mould (h = 6 mm and ϕ = 4 mm) according to an ISO standard 9917-1 [22] and exposed to LED light for 40 s on each side. After considering the above physical properties, 0% and 15% samples were chosen for mechanical testing. Prepared bar specimens were positioned on an Instron 8871 machine (MA, USA) with a 10,000N load cell at a crosshead speed of 1.0 mm/min [23]. The Vickers hardness (HM-221, Mitutoyo, Tokyo, Japan) was measured with 300 gf (2.94 N) for 20 s in three different spots on each specimen, and these values were averaged (n=10). Lastly, flowability was measured using 20, 50, or 75 μm wide grooves, replicated in silicon mold using metal apparatus for reproduction of detail (ISO 6873 for dental gypsum). After filling above each groove, resin-silicon was perpendicularly sectioned and investigated by optical microscope to check filling ability. Continuous contact between resin and grooves were optically investigated and continuous contact was marked as characteristics of successful flowability.

Surface characteristics of dental sealant

The surface of the specimens were analyzed using scanning electron microscopy (SEM, Sigma 500; ZEISS, Oberkochen, Germany) and and surface profiler (n = 10, Ra, SJ-400, Mitutoyo, Japan) respectively as described in detail elsewhere [24-26].

Cytotoxicity test

Immortalized human oral keratinocytes (IHOKs) were used in this study [27]. A cytotoxicity test was performed based on an ISO standard [28]. After cells were seeded (1×10^4 cells/ 96-well plate) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, one

part of the extract from the specimens was added into one part of Dulbecco's modified eagle medium/nutrient mixture f-12 (3:1) (DMEM/F-12(3:1)) supplemented with 10% foetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). Extracts of specimen (h = 1 mm and ϕ = 15 mm) were obtained at a ratio of 3 cm^2/mL for 24 hr at 37 °C in a shaking incubator (120 rpm) using supplemented media following the recommendations of ISO 10993-12 [29]. After 24 hr of incubation, a water-soluble tetrazolium salt (WST) assay was performed according to previously described methods using light with a wavelength of 450 nm (n=6) [30]. Live (green colored) and dead (red colored) staininng assay (Thermo Fisher Scientific) was performed according to manufacturer's instruction to confirm above WST assay.

Estrogenicity assay

Estrogenicity was investigated by quantifying newly synthesized DNA of human MCF7, an established estrogenic cell line endogenously expressing estrogenic receptor α, for 24 hr using nucleoside analogue bromo-deoxyuridine (BrdU) and Click-iT™ Plus EdU Flow Cytometry Assay Kits (Thermo Fisher Scientific) according to modified procedures [31]. MCF-7 was cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (PS) at 37 °C in an atmosphere of 5% CO_2 and 95% air under saturating humidity. MCF-7 cells were seeded in 24-well plates to an initial concentration of 20,000 cells per well in DMEM with 10% FBS and 1% PS (1 mL/well). After 24 hr of cell adhesion, the cells were washed with phosphate-buffered saline (PBS), and the culture medium was changed to DMEM supplemented with 10% synthetic knockout serum replacement (growth factor and steroid-free) [32] and 1% PS without phenol red, consisting of 200 mM L-glutamine, 1 M hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer, 100 mM sodium pyruvate and 1%

of 10 mg/mL penicillin-streptomycin (refer to supplemented steroid-free media). Extract from each specimen in cell culture medium supplemented with steroid-free at a ratio of 3 cm^2/mL was added to MCF-7, and the samples were cultured for the next 72 hrs. Extract was performed at 37 °C for 24 hr under shaking condition (180 rpm). Positive and negative controls were 1×10^{-8} M 17-β-estradiol (Sigma) and 10 nM bisphenol A (Sigma), and steroid-free medium, respectively. Fluorescence-activated cell sorting (FACS) was performed according to the manufacturer's protocol. Briefly, BrdU-treated cells were fixed, permeabilized, and then labelled with fluorescein. A FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation/emission wavelengths of 408/530 nm was used for analysis. Data for 10,000 cells in each sample (n = 3) were analysed by CellQuest Pro software (v.5.1 BD Biosciences).

Statistical analysis

The data are expressed as the mean ± SD of at least three independent experiments. Statistical significance was evaluated by a one-way analysis of variance with a Tukey post hoc test using SPSS (Version 21.0; SPSS, Chicago, IL) when the equality of variance among groups was met. When equality of variance among groups was not satisfied, Welch test with Dunnett's T3 as post hoc test was used. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of isosorbide-derived biomonomer

Light-curable ISDBs have been newly synthesized to replace the BPA-based Bis-GMA. Viscocity of ISDB was measured as 2.42 Pa·s while that of Bis-GMA and TEGDMA was 473 and 0.01 Pa·s respectively under the same conditions. After ISDB was purified by column chromatography, it was chemically-characterized by ^{1}H NMR spectrometer, a mass spectrometer, and an elemental analyser, presenting the designed chemical structure of BHIS and ISDB (**Appendix Fig. 1**). After the experimental dental sealant was combined with TEGDMA (matrix) and CQ/EDMAB (photo-initiator) to form a model dental restorative material, light-curable polymerization was checked by FT-IR, which revealed a decrease in C=C (1637 cm^{-1}) content over the light curing time (**Appendix Fig. 2**). Finally, we used GC/MS to determine the possible chemical degradation products of the ISDB-based dental sealant material. Set dental sealant without any filler was aged for 2 weeks in water with enzyme (porcine liver esterase, 10 mg/mL) to mimic the enzymatic hydrolysis in saliva. 2 weeks incubation of specimen with high concentration of esterase was used as an accelerated degradation condition, resulting in severe degradation of biopolymer due to their hydrolysing capacity against the ester bonds of polymer, which is a major mechanism for biopolymer enzymatic depolymerisation in vivo condition [33]. Isosorbide derivatives, including an ethylene glycol derivative ((3*R*,3a*R*,6*S*,6a*R*)-6-(2-hydroxyethoxy)hexahydrofuro[3,2-*b*]furan-3-ol (isosorbide-ethylene glycol)), were detected and have not been identified as estrogenicity inducers so far (**Appendix Fig. 3)**. The peaks at 2.39 and 5.82 min in the GC chromatogram and their corresponding mass spectrum peaks (maxima at 193.23 and 192.62) corresponded to the molecular weight (~193) of isosorbide-ethylene glycol and its isomer (**Fig. 1B**).

Characterization of ISDB-based dental sealant

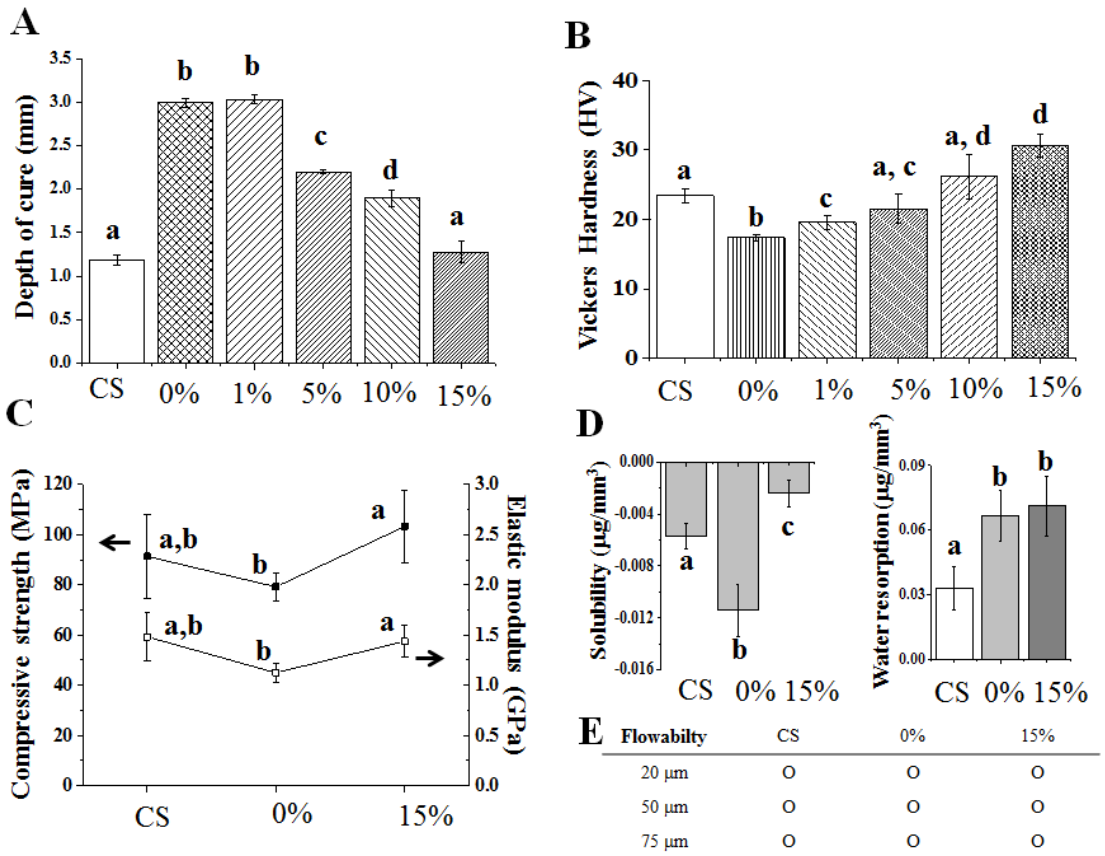


Figure 2. Physico-chemical properties of ISDB-based dental materials. (A) Depth of cure (n=5), (B) Vickers hardness (n=10), (C) compressive strength and modulus (n=10), (D) water solubility/resorption (n=5), and (E) flowability into 25, 50 and 75 μm gaps (n=5). Different letters indicate statistically significant differences between their corresponding values (P < 0.05). CS, 0% and 15% mean commercial sealant (Concise), ISDB sealant without filler and ISDB sealant with 15 wt% filler, respectively.

As a control group, commercially available dental sealant (Concise™, CS), having 5~10 wt% filler, was selected. The depths of cure all reached over 1.5 mm and increased in the order CS=15% < 10% < 5% < 1% < 0% (**Fig. 2A**). Vickers hardness increased with increasing filler amount (17.4 ± 0.4 ~ 30.6 ± 1.7 HV), and these values were significantly increased by 15% compared with their values of CS (**Fig. 2B,** P<0.05, versus 23.4 ± 1.0 HV). The compressive strength and elastic modulus from 15% and CS samples showed comparable values (91.3 ± 16.6 MPa versus 103.2 ± 14.5 MPa and 1.48 ± 0.23 GPa versus 1.43 ± 0.15

GPa at P>0.05,), while these properties were both greater in the 15% sample compared to the 0% sample (**Fig. 2C,** P<0.05). The water solubility of 15% and CS samples was ~ -0.006 and ~ -0.002 μg/mm^3, respectively, indicating little difference in solubility (**Fig. 2D,** increase in weight for both materials). Water resorption was slightly increased in 0% and 15% samples compared to the CS samples (**Fig. 2D,** P<0.05) but below the maximum value for dental restorative materials (40 μg/mm^{3,} ISO 4049). Lastly, flowability was measured using 20, 50, and 75 μm wide lines, revealing acceptable flowability to fill pits and fissures (**Fig. 2E)**. Scanning electron microscopy (SEM) images after 3 weeks of DW incubation at 37 °C showed a surface similarly roughened (~ 2-fold) to surfaces before incubation due to the degradation of polymer in all groups, which was supported by parameters from roughening analysis (**Fig. 3A and B,** Ra, Ry, and Rz).

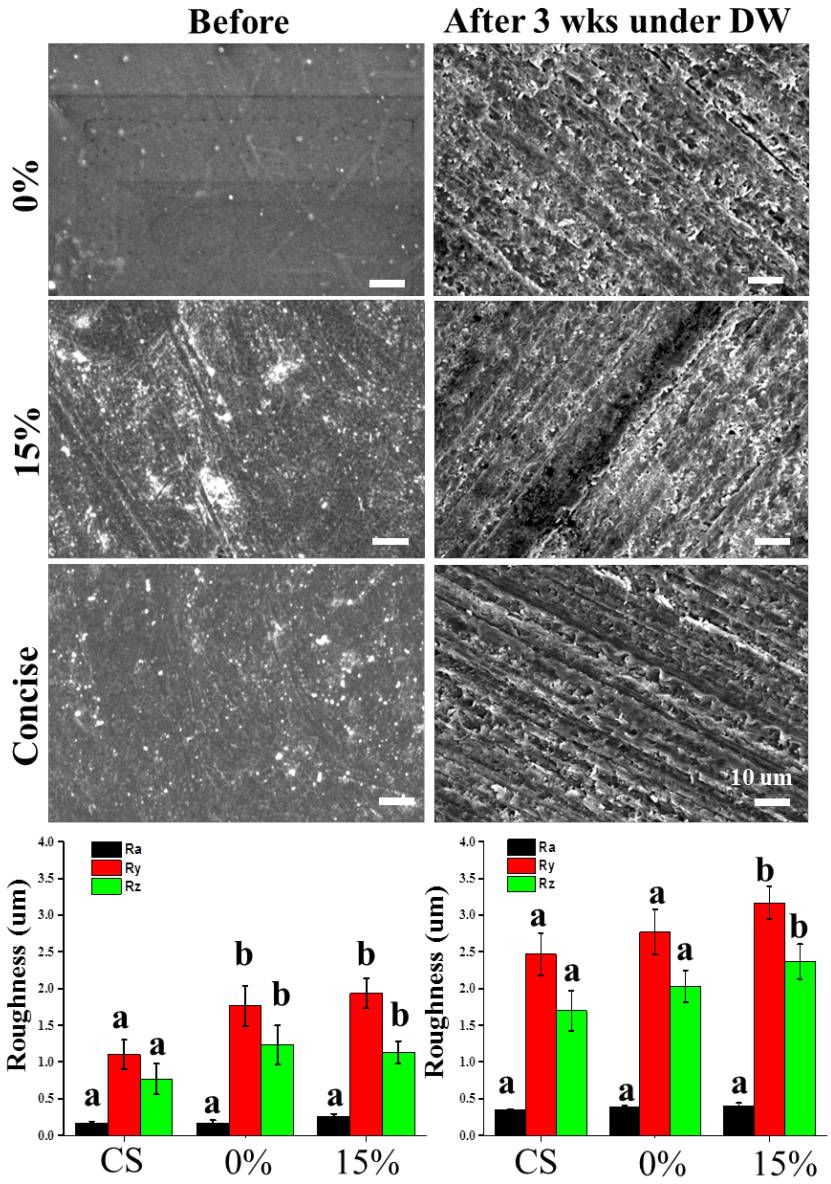


Figure 3. Surface morphology and roughness before and after incubation in distilled water for 3 weeks. All experimental groups showed surfaces that had increased in roughness (~ two-fold) after long incubation times, as quantified by SEM images and roughness analysis. CS, 0% and 15% mean commercial sealant (Concise), ISDB sealant without filler and ISDB sealant with 15 wt% filler, respectively. Different letters indicate statistically significant differences between their corresponding values ($P < 0.05$).

Cytocompatibility test

The cell viability of oral keratinocytes, the major cell type in the outermost layer of oral

mucosa, against 12.5~100% extract was comparable among 0%, 15% and CS samples (**Fig. 4A**, $P > 0.05$). This result was also visualized in images of live and dead cells with 100% extract (**Fig. 4B**), which showed similarly numbers of live cells in all groups compared to the control, which was not treated with an extract.

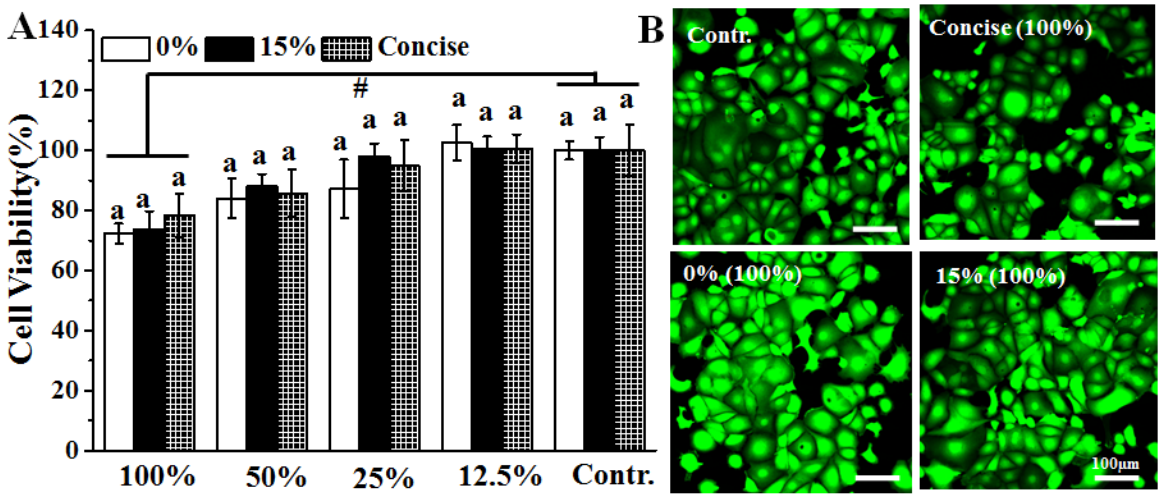


Figure 4. Cytocompatibility of ISDB-based restorative materials with human oral keratinocytes. Cytotoxicity test using extracts (24 hr at 37 °C) from specimens was performed by (A) WST and (B) live (green) and dead (red, rarely detected due to detachment of dead cells during washing) assays (n=6). The # sign indicates a statistically significant difference between the 100% extract and the control samples ($P < 0.05$). Experimental groups exhibited comparable cytotoxicity.

Estrogenicity

In vitro estrogenicity was investigated with estrogen-sensitive MCF-7 cells. DNA synthesis of MCF-7 cells, measured by the BrdU assay, was not significantly increased among 0%, 15% and CS samples (**Fig. 5**, $P > 0.05$), while positive controls (10 nM BPA and 100 nM extradiol) showed an increase in DNA synthesis in MCF-7 cells ($P < 0.05$).

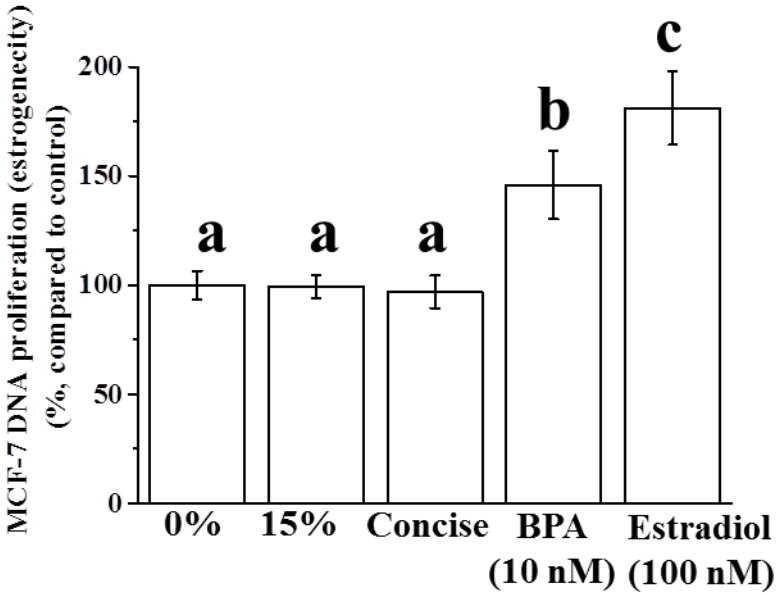


Figure 5. Results of the estrogenicity assay monitoring DNA synthesis for 24 hr as an estrogen-mimicking action. Note the increased proliferation of positive groups (BPA and estradiol) relative to the control for estrogenicity-sensitive MCF-7 cells, which indicates an estrogen response. The estrogen response was not detected from 0%, 15% and Concise samples, revealing no estrogenicity.

Discussion

In the present study, we report the successful synthesis and characterization of ISDBs for use in medical device (i.e. dental restorative, medical implant, etc.). Among various medical devices, dental sealant from dental restorative materials is chosen due to their common use for children to prevent dental caries and many worries about estrogenicity from them. Here, ISDBs was utilized as a replacement of dental sealant, as a model, for oil-derived Bis-GMA to address the problem of estrogenicity. We synthesized ISDBs from isosorbide to produce C=C bonds on both sides of the monomers for polymerization with other monomers such as TEGDMA via an addition reaction. Viscosity of ISDB (2.42 Pa·s) was 200 times less than that of Bis-GMA (473 Pa·s) and 24 times higher than that of TEGDMA (0.01 Pa·s) under the same conditions, which possibily increase workability and adaptation of dental sealant on pit

and fissure when ISDBs were adapted as major components [34]. ISDBs were utilized to fabricate experimental dental sealant as a model of ISDB-based restorative dental material, and they were further supplemented with silanized micro-silica powder at levels up to 15 wt% to generate physico-mechanical properties comparable with those of a conventional dental sealant (i.e., CS). The optimized light curing time (40 s) was determined based on the C=C (1638 cm^{-1}) content decrease and depth of cure increase with increasing light curing time (not shown). The depth of cure, compressive strength, and water resorption/solubility were measured based on the ISO standards for polymer-based restorative materials. Although the standard of dental sealant required only depth of cure as a physico-chemical property, one of the basic parameters related to polymer-based restorative materials (water resorption/solubility) and the resistance to biting force (compressive strength) were chosen. The measured of depth of cure, water resorption/solubility and compressive strength values were comparable between 15% filler ISDB and CS samples, while Vickers hardness was ~40% greater in 15% filler ISDB samples than in CS samples. To investigate in detail the surface degradation in the oral cavity, which is an indicator used to minimize plaque formation on the restorative surface and consequent initial/secondary dental caries, the specimen was incubated under DW for 21 d at body temperature (37 °C) with 100% humidity and dried at 60°C every day. The original smooth surface morphology increased in roughness ~ 2-fold in 0% and 15% filler ISDB and CS samples, revealing comparable surface degradation between ISDB-based restorative materials and commercial Bis-GMA-based materials and raising the possibility of cytotoxic or other biological concerns (estrogenicity) from degraded byproducts/monomers.

Similar to the commonly used Bis-GMA-based dental restorative materials, which are used due to their desired mechanical properties, the polymerization of ISDB-based composites is

never complete, and thus unreacted monomers can remain after the curing process and induce adverse biological effects [7]. To investigate the initial adverse effects which may be strongly induced by release of unreacted monomers, a cytotoxicity test for human oral keratinocytes, a representative cell type in the outermost layers of the oral mucosa, was performed with the extracts; this test revealed comparable cytotoxicity between the commercial Bis-GMA-based dental material and the ISDB-based material.

Risks of safety issues arising after hydrolysis of the materials by enzymes (i.e., esterase) in the oral cavity remain. In the case of the Bis-GMA dental composite, hydrolysis occurs in vitro by saliva enzymes (i.e., esterase) at its ester bond (O=C-O), and byproducts such as Bis-GMA without 1~2 methacrylic acid groups were released without estrogenic BPA [35], indicating a low possibility of estrogenicity [7]. However, health concerns might arise with respect to Bis-GMA-derived degradation products, since they all contain the BPA moiety, and in vivo conditions might accelerate hydrolytic degradation to less reactive ether bonds (C-O-C), which is supported by clinical investigations with high concentrations of BPA in saliva from dental sealant-treated children [8,9,36,37]. To tackle this BPA-induced estrogenicity, we designed a BPA-free ISDB-based dental material. As designed, when degradation of the ISDB-based dental restorative materials was tested with esterase to mimic hydrolytic degradation activity in the oral cavity, only the isosorbide derivate (isosorbide-ethylene glycol) without a BPA moiety was detected; this derivate has not been identified as a strongly toxic compound or an estrogenicity inducer so far, which was confirmed by no estrogenic response from ISDBs dental sealant in current in vitro study. The estrogen response was not either detected for Concise samples, meaning that fully polymerized Bis-GMA based dental materails can’t generate estrogenic response under in vitro condition [38]. According to other literatures, ISDB-based biopolymers have been considered to generate a

noncytotoxic and non-inflammatory response compared to the culture-grade polystyrene control [13,39]. Even though the estrogenicity of isosorbide-based biopolymers and their derivatives have not been reported until now, further study investigating the safe use of ISDBs in dental restorative materials is necessary for clinical application.

In summary, ISDB-based biopolymers are highlighted as possible replacement biomonomers due to their safe origin, biocompatibility, comparable physico-mechanical properties and lack of estrogenicity compared to petrochemically derived Bis-GMA materials [12,18]. Along with the above benefits reported in the current investigation and the literature, this study describes the first trial utilizing ISDBs in dental restorative materials.

In conclusion, this study is the first to demonstrate the synthesis of ISDBs and their possible utilization in dental restorative materials. Within the limitations of this study, ISDBs had physico-chemical and biological characteristics comparable to those of commercial Bis-GMA-based restorative materials. With the design of a BPA-free polymer structure/network and results from in vitro degradation and estrogenicity tests to confirm the absence of BPA as a hydrolytic byproduct and its consequent estrogenicity, ISDB can be used in dental restorative materials and further studied in in vivo biocompatibility tests and clinical trials.

Author contributions

SK Jun contributed to the conception and design of the study, specimen preparation, and data acquisition (mechanical properties), analysis (mechanical properties), and interpretation; JR-Cha contributed to the conception and design of the study and data acquisition (biopolymer), analysis (biopolymer), and interpretation; HW Kim and JC Knowles contributed to material, biological and data analysis and critically revised the manuscript; and JH Lee and HH Lee equally contributed to the conception and design of the study, data analysis and interpretation,

and critical revision of the manuscript. All authors have approved and agreed to be accountable for all aspects of this work.

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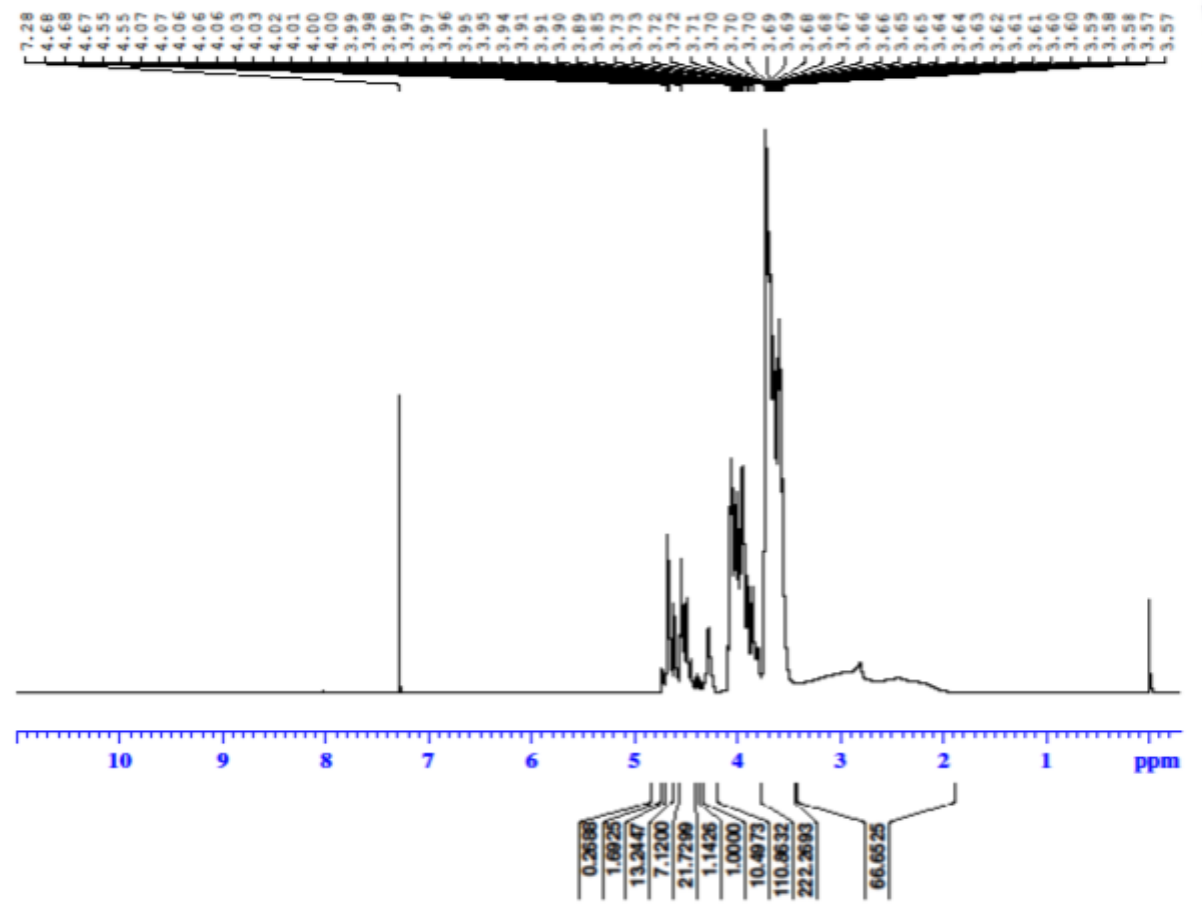
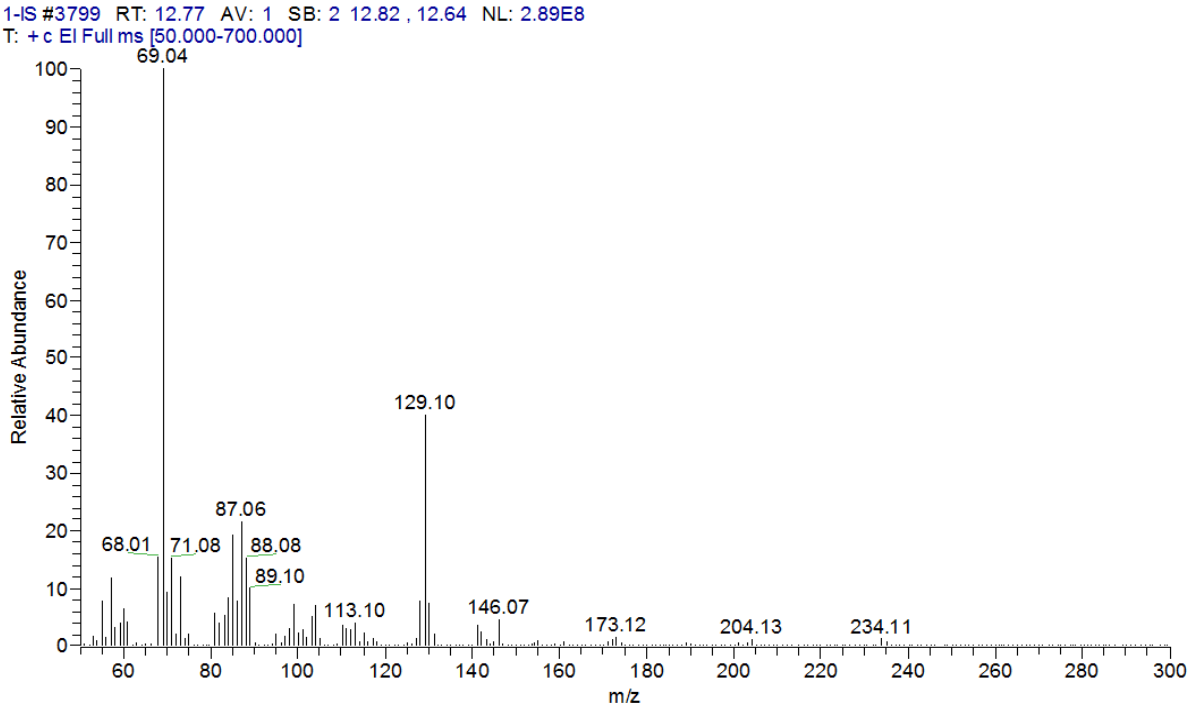
Appendix

sTable 1. Summary of NMR analysis from BHIS and ISDB

Compound	Mass spectra positive ion mode (found) $[M]^{+\cdot}$	EA calculated (found), % C	H	N	O	^{1}H NMR ($CDCl_3$) δ (ppm)
BHIS ($C_{10}H_{18}O_6$)	234.11	48.43	7.83	-	43.68	2.00-3.40 (s, 2H hydroxy) 3.57-3.73 (m, 8H, ethyl) 3.85-4.07 (m, 4H, isosorbide) 4.54-4.74 (m, 4H, isosorbide)
ISDB ($C_{24}H_{36}N_2O_{12}$)	544.55	52.73	6.88	5.17	35.28	1.95 (s, 6H methyl) 3.51 (s, 4H ethyl) 3.60-3.67 (m, 6H ethyl) 3,87-4.05 (m, 6H isosorbide) 4.24 (s, 6H ethyl) 4.50-4.64 (m, 2H isosorbide) 5.1 (s, 2H amine) 5.60 (s, 2H acryl) 6.12 (s, 2H acryl)

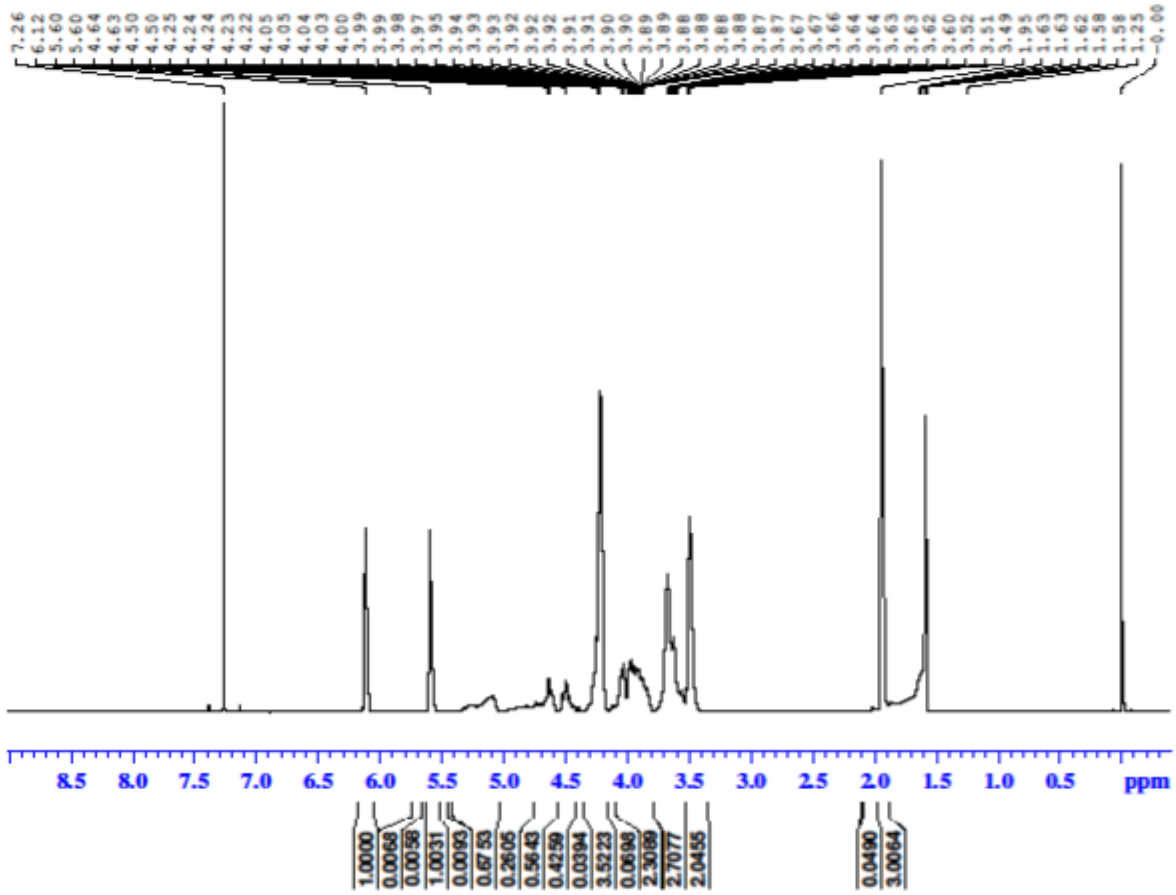
A. BHIS (intermediate product)

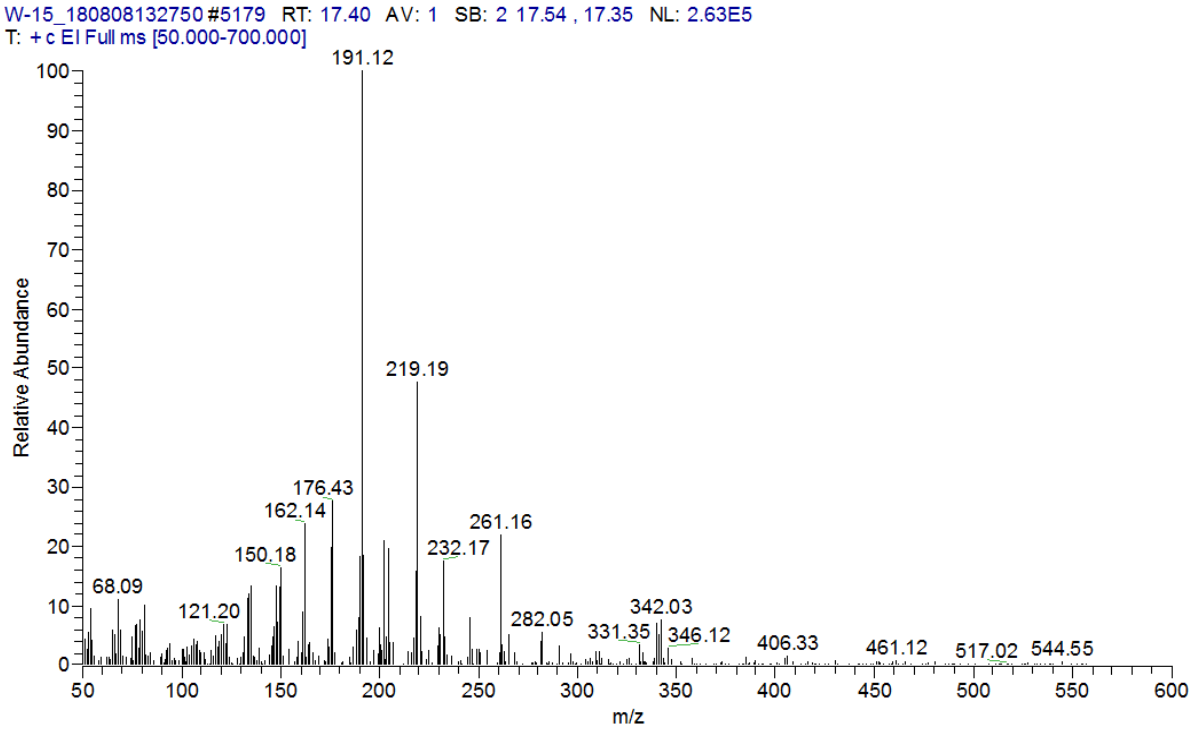




sFig. 1 continued

B. SCDB (final monomer)



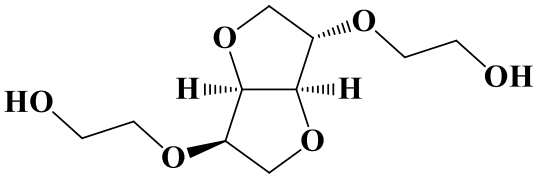


sFig. 1 continued

C. Element analysis and designed chemical formula

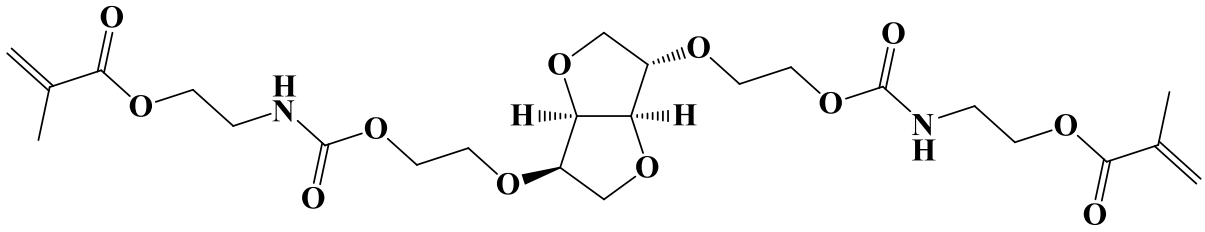
Sample name	Nitrogen	Carbon	Hydrogen	Oxygen
BHIS	n.d.	48.4326	7.8312	43.6757
ISDB	5.1731	52.7328	6.8824	35.2804

BHIS



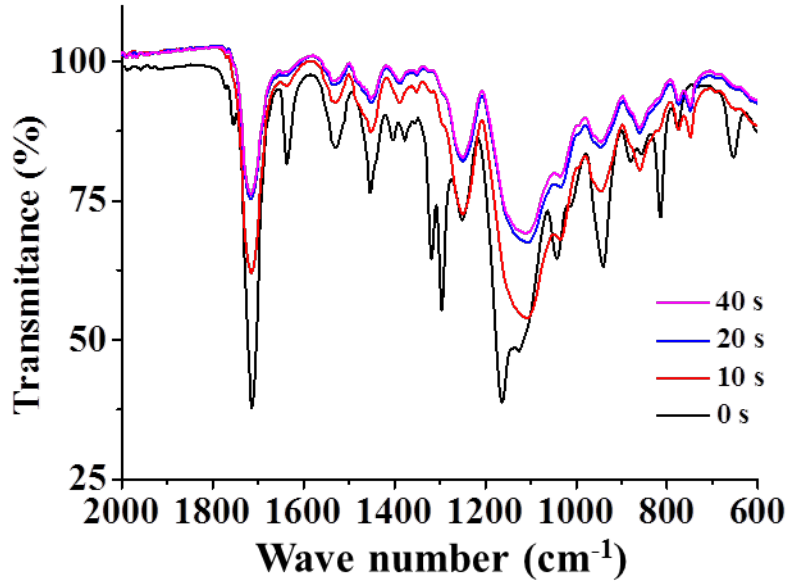
Chemical Formula: $C_{10}H_{18}O_6$
Exact Mass: 234.11
Molecular Weight: 234.25
m/z: 234.11 (100.0%), 235.11 (11.0%), 236.11 (1.2%)
Elemental Analysis: C, 51.27; H, 7.75; O, 40.98

CSMA

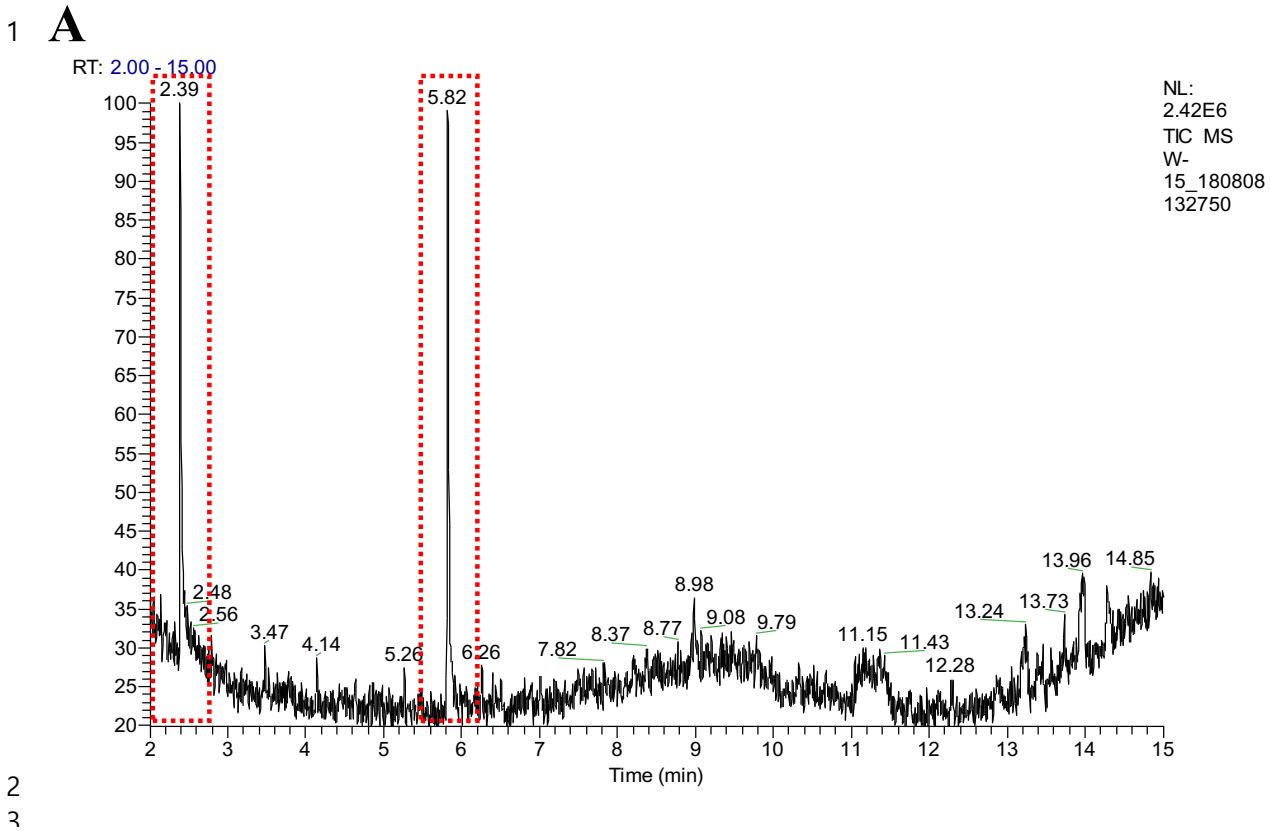


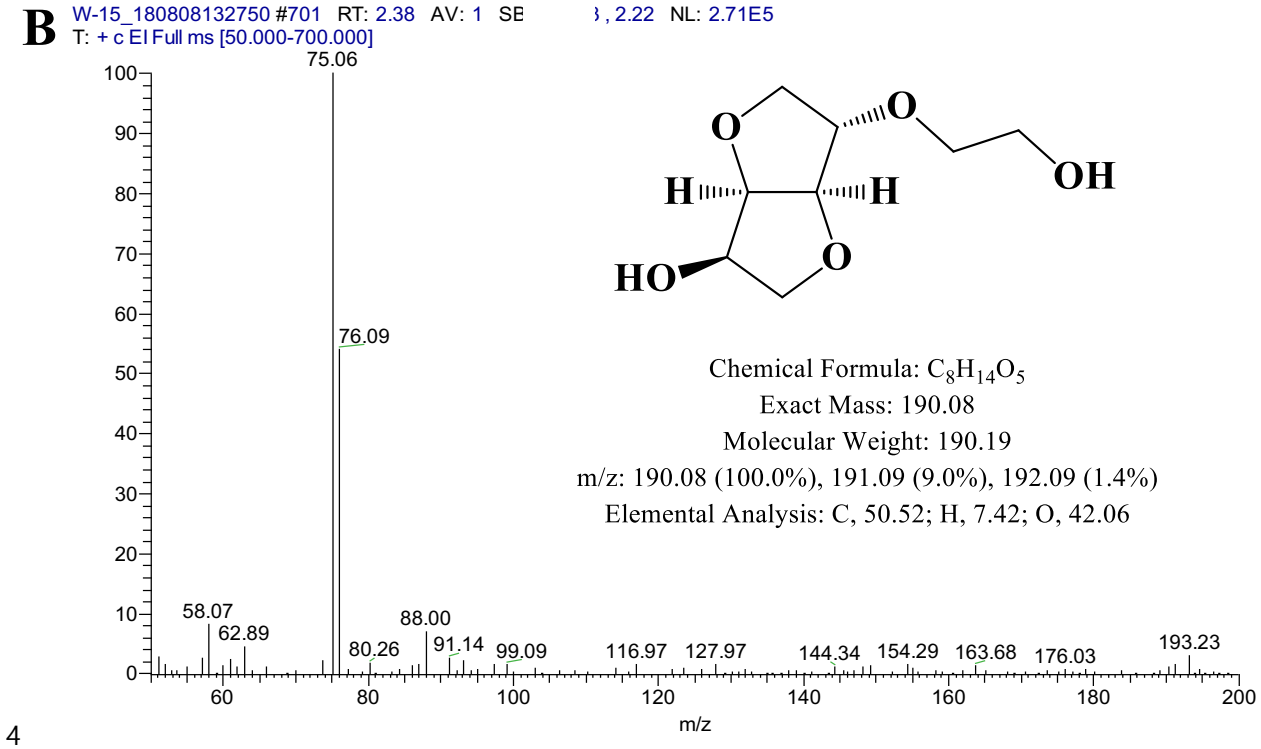
Chemical Formula: $C_{24}H_{36}N_2O_{12}$
Exact Mass: 544.23
Molecular Weight: 544.55
m/z: 544.23 (100.0%), 545.23 (26.8%), 546.23 (6.0%)
Elemental Analysis: C, 52.93; H, 6.66; N, 5.14; O, 35.26

sFig. 1 Fabrication of (A) BHIS and (B) Bis-GMA-free monomer (ISDB) and their characterization by NMR (up) & GC/MS (down). (C) Analysed element of each product and their designated chemical formula.



sFig. 2 FT-IR spectra of ISDB-based sealant vs. light curing time

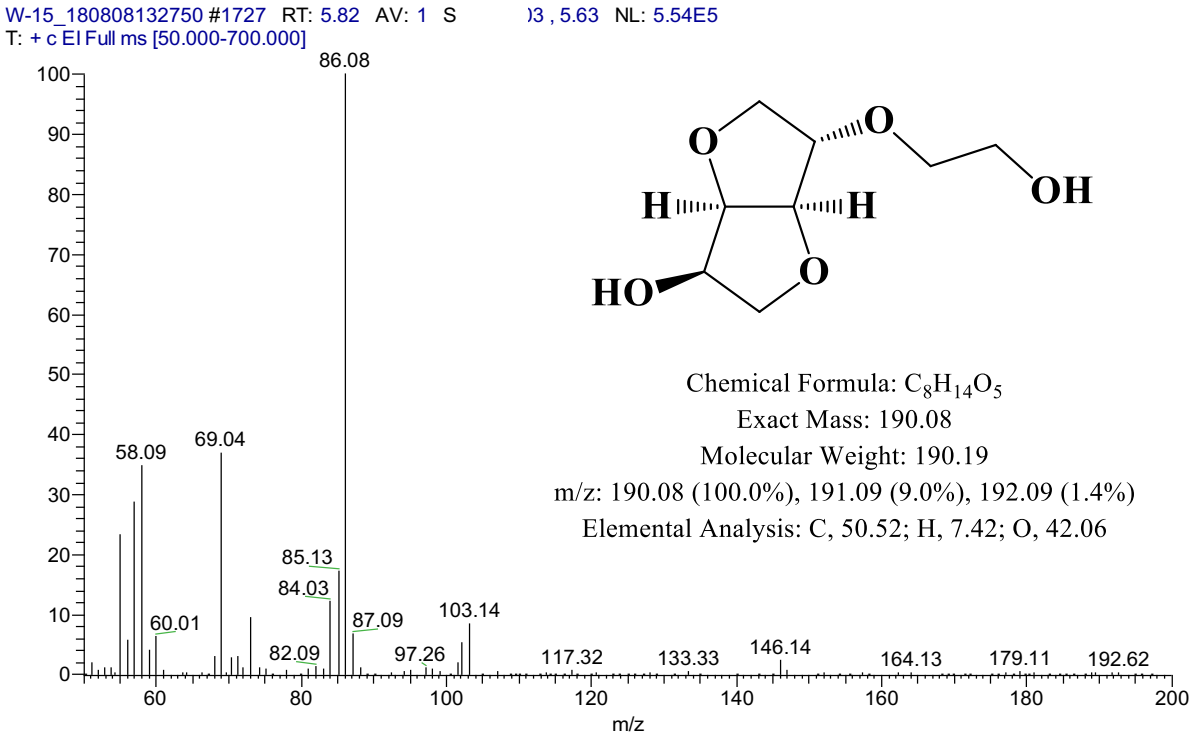




Chemical Formula: $C_8H_{14}O_5$
Exact Mass: 190.08
Molecular Weight: 190.19
m/z: 190.08 (100.0%), 191.09 (9.0%), 192.09 (1.4%)
Elemental Analysis: C, 50.52; H, 7.42; O, 42.06

sFig. 3 continued

C



sFig. 3. GC/MS spectra and retention times of ISDB degraded by esterase: (A) full-scan spectrum, (B) spectrum at 2.39 min, (C) spectrum at 5.82 min.