3.1 Materials

In this study, the materials listed in Table 3.1 were used as received, without further purification.

Table 3.1 Materials used in the study

Materials	Acronym	Source	Purity %	LOT NO.	Function
Alkyd polyol	AlkOA65N	Chemistry Departmen, UM, Malaysia	-	-	Hydroxyl groups carrier for urethane formation.
Polymeric methylene diphenyl diisocyanate	MDI	Yantai Wanhua Wannate, China	95	-	Isocyanate groups carrier for urethane formation
2-Hydroxyethyl methacrylate	HEMA	Esstech, USA.	99	614-22	Urethane formation and acrylate endings.
Dibutyltin dilaurate	DBTDL	Sigma-Aldrich	99	1293912	Urethane catalyst
Hydroquinone	HQ	Merck, Germany	99	8.22333. 0250	Stabilizer (inhibitors)
Bis-phenol A glycidyl dimethacrylate	Bis-GMA	Esstech, USA.	93	582-44-03	Monomer used as the control
Triethylene glycol dimethacrylate	TEGDMA	Sigma-Aldrich, Germany	95	07709K E-067	Co-monomer
Ethoxylated Bisphenol A dimethacrylate	Bis-EMA	Esstech, USA.	98	597-09-14	Co-monomer
Camphoroquinone	CQ	Sigma-Aldrich, Germany	97	124893	Light-initiator
Ethyl-4-N,N-dimethyl amino-benzoate	4EDMAB	Sigma-Aldrich, USA	98	39120	Light-activator (reducing agent)
Silanated barium borosilica glass	-	Esstech, USA.	-	644-30	Filler

3.2 Synthesis of experimental monomer

Alkyd polyol AlkOA65N (Figure 3.1) was selected for synthesis of the experimental monomer. A preliminary study was done and this polyol was selected from three polyols, based on palm oil (Appendix I). This polyol was synthesized from the reaction of oleic acid, glycerine and phthalic anhydride. The full details on synthesis, reactions, and properties are illustrated in Appendix II.

Figure 3.1 Chemical structure of AlkOA65N polyol

The synthesis of the experimental urethane acrylate macromer (UAM) was carried out in two steps. The first step was the reaction of excess amounts of polymeric methylene diphenyl diisocyanate (MDI) with AlkOA65N to produce urethane prepolymer with terminal –NCO group (Figure 3.2).

Fifty g of MDI was poured into a 500 ml rounded flask and purged with nitrogen for 5 minutes to expel all moisture from inside the flask. The flask was heated to 60°C in a temperature controlled water bath. Twenty six g of AlkOA65N was added drop by drop to the MDI within a 15 minute time frame, using a dropping funnel under nitrogen atmosphere. The mixture was then stirred for 30 minutes at a constant temperature of 60

°C, using a mechanical stirrer at 150 ± 50 rpm. This procedure was carried out to ensure that all the hydroxyl groups of AlkOA65N reacted with the highly reactive aromatic MDI to form urethane prepolymer.

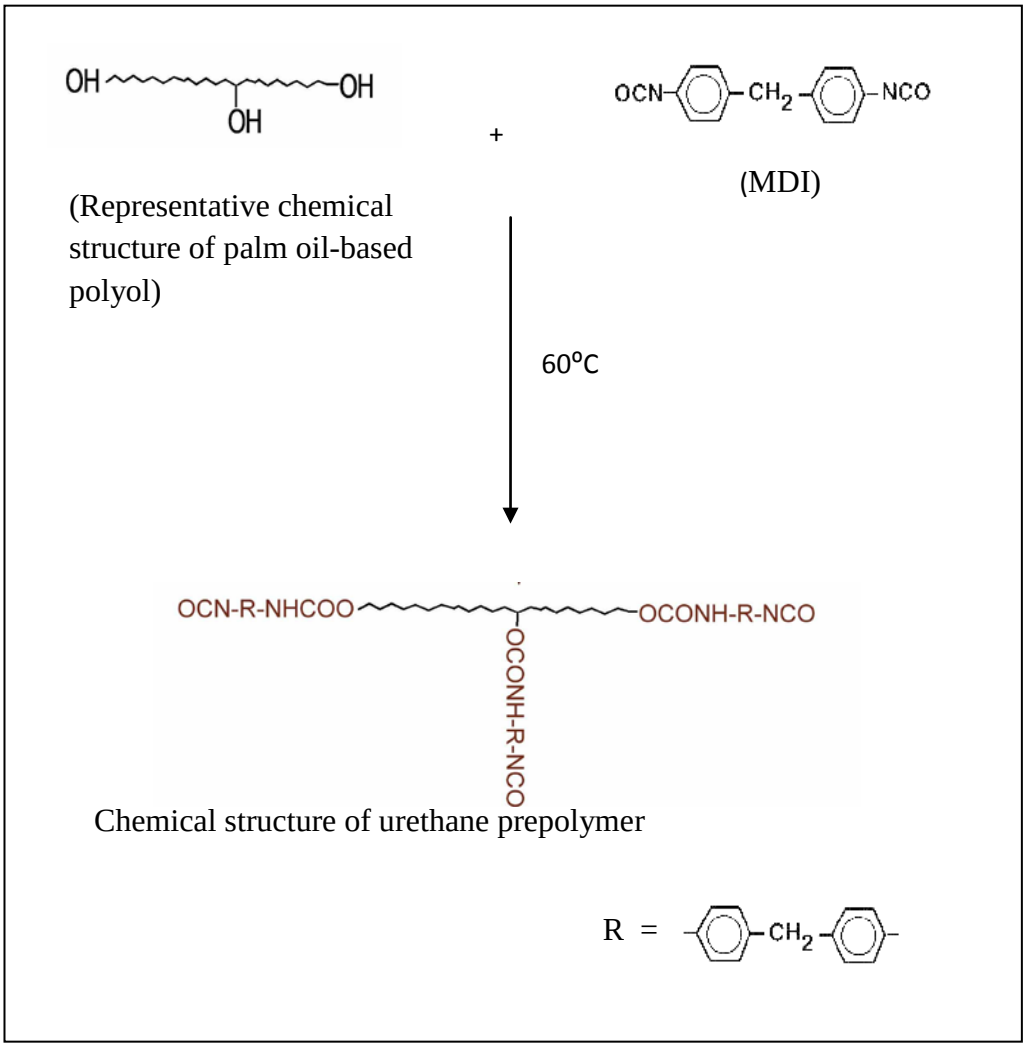


Figure 3.2 Reaction route of UAM synthesis (first step)

The second step involved the reaction of urethane prepolymer with an acrylic monomer, in order to obtain urethane acrylate macromer (Figure 3.3). The mixture was cooled down by adding ice cubes into the water bath until the temperature dropped to 10 °C. While, this mixture was cooling down, a mixture of 90 g of 2-hydroxyethyl methacrylate (HEMA) and 0.1 g of hydroquinon (HQ) was mixed with 0.05 g dibutyltin

dilaurate (DBTD) as a catalyst. This mixture was then poured into a dropping funnel and added drop by drop into the flask within a 30-40 minutes time frame. The mixture in the flask was stirred constantly at 160 rpm. The stirring was continued for 2 hours to ensure that the reaction was completed and UAM formed. The entire synthesis was carried out under a nitrogen blanket, as is shown in (Figure 3.4). This was done to avoid byproduct formation due to moisture contamination as isocyanate groups of MDI showed high reactivity.

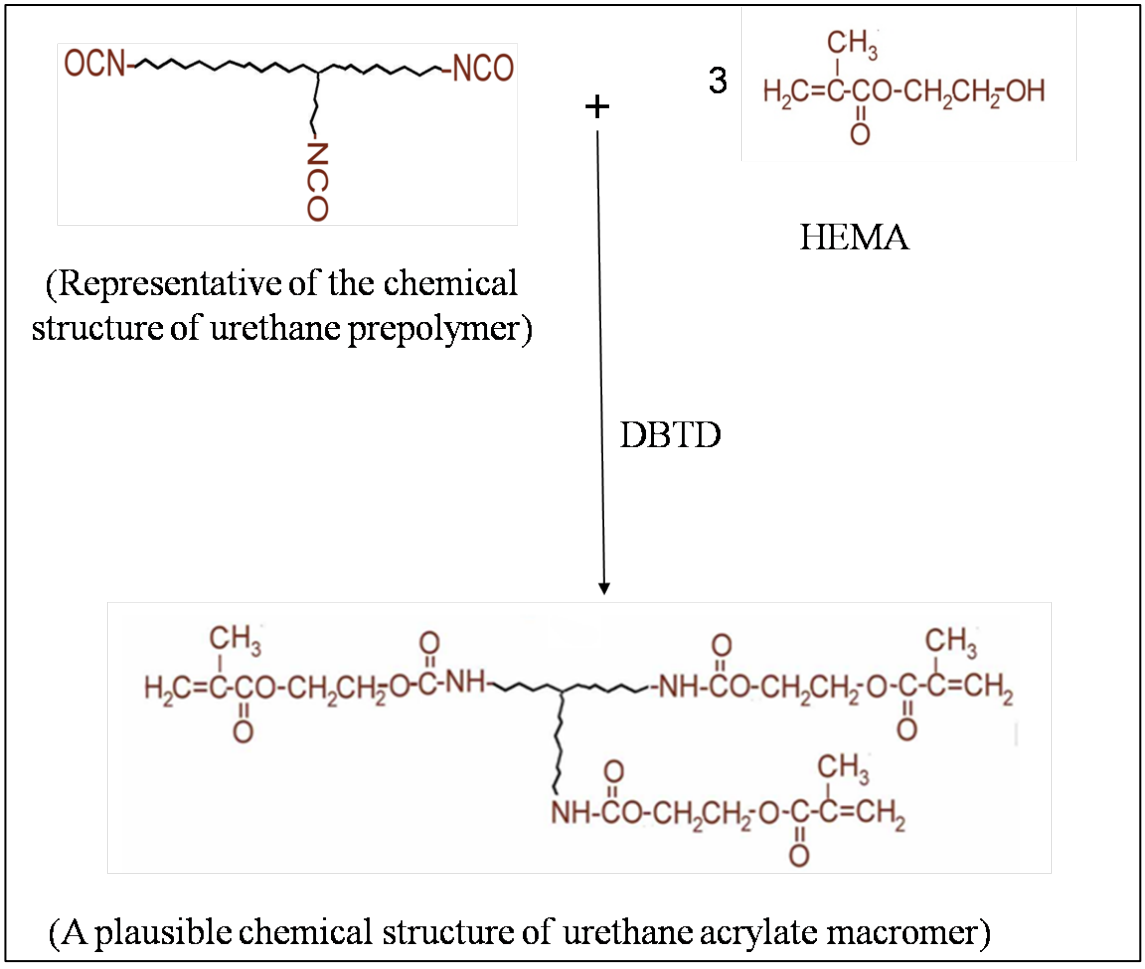


Figure 3.3 Reaction route of UAM synthesis (second step)

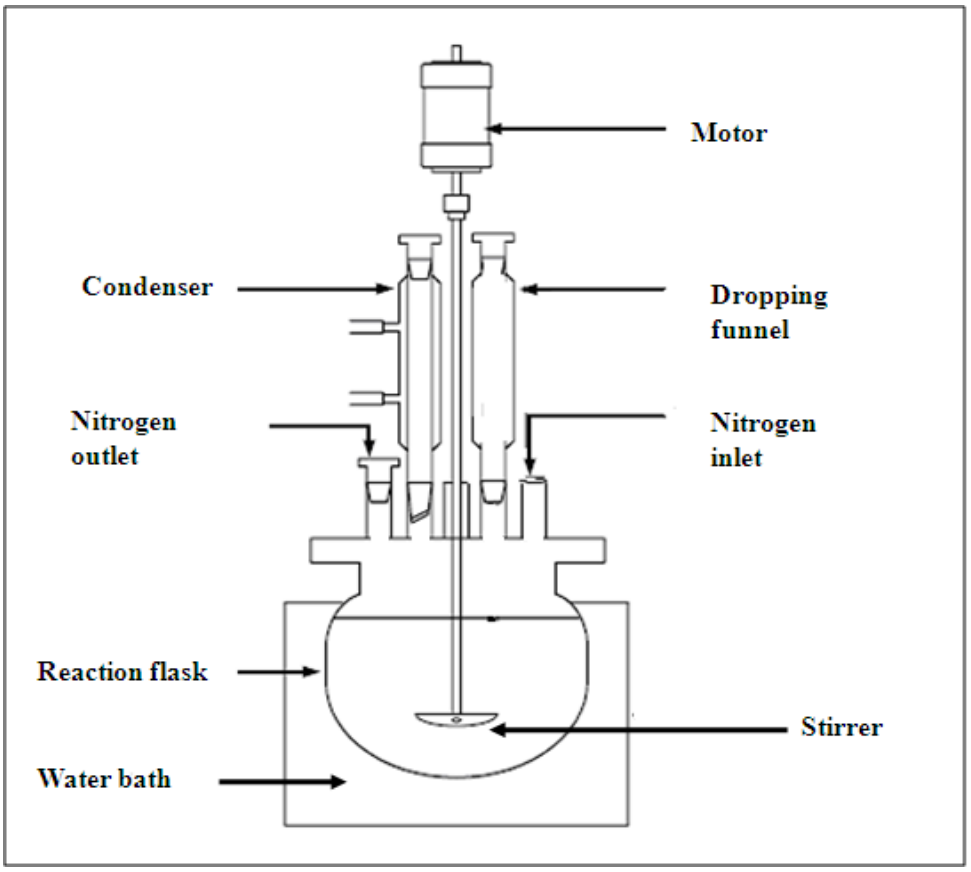


Figure 3.4 The reaction set up

Fourier transform infrared spectroscopy (FT-IR) (Perkin Elmer, spectrum 2000, USA) (Figure 3.5) was used to confirm the formation of UAM. In order to obtain the FT-IR spectrum, one drop of UAM was smeared over a NaCl crystal window (Figure 3.6A) and mounted in the FT-IR chamber using a holder (Figure 3.6B). The FT-IR spectrum was scanned in the range of 370-4000 cm^{-1} wave number range, for 16 scans, at 4 cm^{-1} resolution. In addition, the same procedure was carried out to obtain the FT-IR spectra of the starting materials MDI and AlkOA65N. The remainder UAM was poured into a dark glass container and stored at 4°C until further use.

Figure 3.5 FT-IR spectroscopy used in this study

(A)

(B)

Figure 3.6 FT-IR accessories

(A) NaCl crystal window
(B) Holder

3.3 Experimental resins

3.3.1 Preparation of light-curable resins

Two experimental resins were prepared consisting of the synthesized experimental UAM and commercially obtained Bis-GMA, which is a common resin used for dental composite formulation.

Camphoroquinone (CQ) and ethyl-4-N,N-dimethylamino-benzoate (4EDAM) were used as a light-initiator system to prepare the light curable experimental resin for both Bis-GMA and experimental UAM, which were coded B and U respectively.

0.2% (weight) CQ and 0.8 % (weight) 4EDMAB were weighed using an analytical balance (Sartorius, AG Gottingen, Germany). The CQ and 4EDMAB were then mixed with each monomer (99% by weight) in a glass container and manually stirred using a plastic spatula for 15 minutes. The mixture was then left to rest for 24 hours to ensure that the light-initiator particles had dissolved completely in the monomer. Each light-curable resin was then filled into disposable plastic syringes and wrapped with black tape and coded according to their monomer type. The whole procedure was carried out under yellow light to avoid the premature polymerization of the experimental light-curable resins.

Prior to storage, a moderate vacuuming to 7 mbar was applied using a vacuum pump (Vacuubrand, Germany), for 5 hours to eliminate any air entrapped during the filling of the syringes (Figure 3.7). These syringes will be used as a delivery device to facilitate handling during the specimen's fabrication in the future. The filled syringes were stored at 4^{o}C for future experiments.

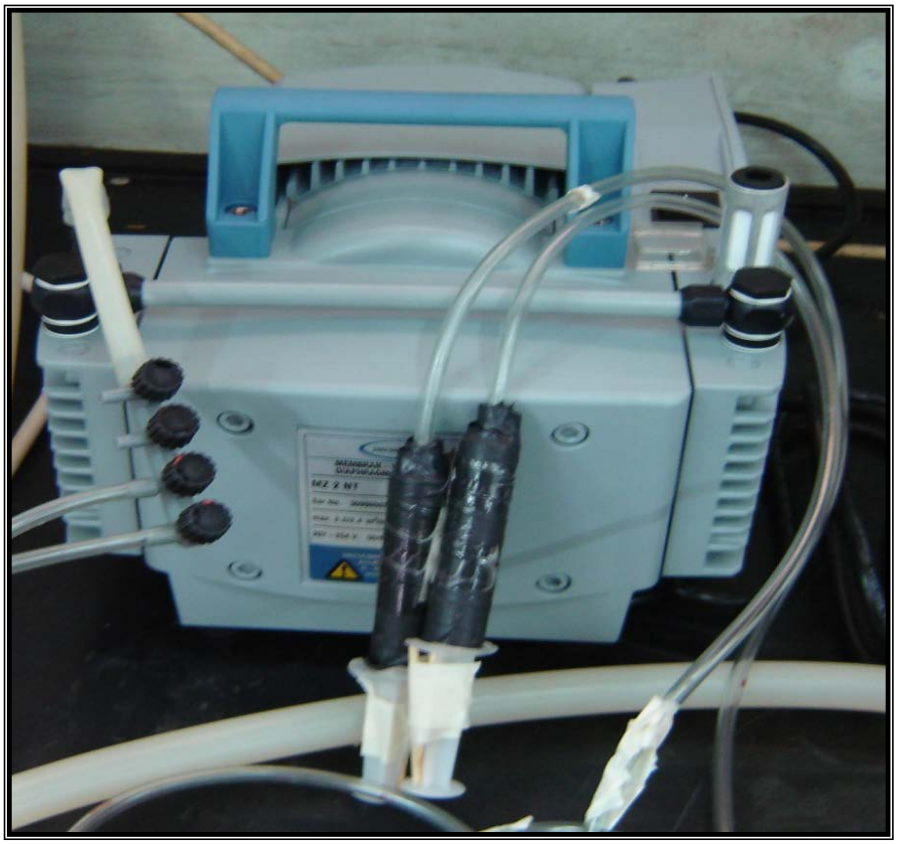


Figure 3.7 A vacuum pump connected with filled syringes

3.3.2 Determination of viscosity

The viscosity of each uncured experimental resin was measured in triplicate (Davity et al., 1998; and Prakki et al., 2007) using a Brookfield viscometer (DV-E, Brookfield Digital Viscometer, USA). The procedure was carried out in accordance to the manufacturers' instructions. First, the chamber of the small adapter (3.8 ml) was filled with monomer, and a spindle (SC4-15) was set up. Then, the small adapter of the viscometer was connected to a water bath (25ºC ± 0.1) as is shown in Figure 3.8. Various spindle speeds were utilized and different viscosity values were obtained, in centipoises (cp). The mean viscosity was then calculated and the same procedure was carried out again for the second and third readings. The whole procedure was carried out under yellow light to avoid the premature polymerization of the uncured experimental resin. For a highly viscous monomer such as Bis-GMA, the monomer was left overnight inside the chamber of the adaptor, to eliminate any air bubbles created during the filling up process of the chamber, which could affect the readings as described in the manufacturer's instructions.

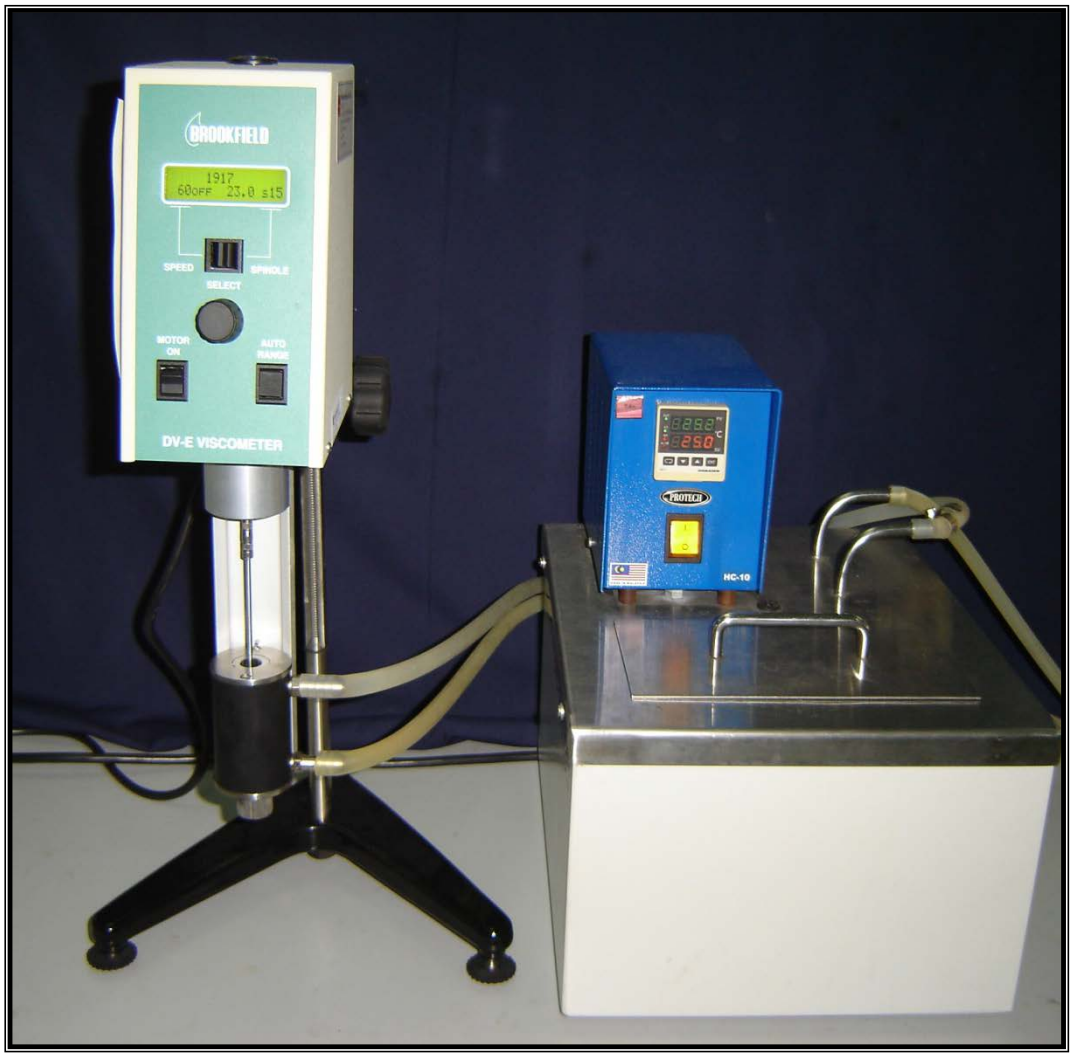


Figure 3.8 A Brookfield viscometer

3.3.3 Determination of degree of conversion and cross-linking density

The degree of conversion (DC) for both experimental resins were determined using the FT-IR spectra of 5 uncured and 5 cured thin films (Peutzfeldt and Asmussen 2005; Lin-Gibson et al., 2006; and Atai et al., 2007). This was done by the identification of the absorption height peaks of aliphatic C=C group at 1636 cm^{-1} and aromatic C=C at 1608 cm^{-1}. The peak height of aromatic C=C showed no change before and after curing (Ferracane and Greener 1984; Atai et al., 2004; Peutzfeldt and Asmussen 2005; Mendes et al., 2005; and Prakki et al., 2007). Thus, percentage of degree of conversion (*% DC*) for each resin can be calculated, based on the change in the ratio of the aliphatic C=C to the aromatic C=C of cured and uncured films, using the following equation:

$$\%\,\boldsymbol{DC} = \left(1 - \frac{A_{1636} \,/\, A_{1608} \text{ of cured film}}{A_{1636} \,/\, A_{1608} \text{ of uncured film}}\right) \times 100$$

where;

A_{1636} is the absorption height peak of aliphatic C=C

A_{1608} is the absorption height peak of aromatic C=C

The FT-IR spectrum of uncured thin film was obtained using the following method. A small drop of the resin was placed between two transparent polyethylene sheets and sandwiched between two glass slides to form a thin film. The total thickness of the uncured materials and sheets was approximately 0.004 mm when measured using a digital caliper (Mitutoyo, Japan), as is shown in Figure 3.9. This film was then fixed in the chamber of the FT-IR spectroscopy. The FT-IR spectrum of uncured film was obtained and the absorption peaks of aliphatic C=C group measured at 1636 cm^{-1} and aromatic C=C group measured at 1608 cm^{-1} were recorded using the software provided

(Spectrum, version 5.3, Perkin Elmer.Inc.) The uncured film was prepared at sub ambient light to avoid polymerization during the procedure.

The FT-IR spectrum of cured thin film was obtained by dropping a small amount of monomer (light-curable monomer) inside the polyethylene sheets, which was sandwiched between two glass slides and irradiated for 60 seconds using light curing unit (Spectrum 800, Dentsply Caulk, USA). The light intensity of light curing unit was 500 mW/cm^2, as checked using a radiometer (Demetron, Model 100, CT, USA). Polyethylene sheets were used instead of NaCl crystals, to avoid resin adhesion on the NaCl crystals window and oxygen inhibition effects (Sideridou et al., 2002).

After one hour of irradiation, the spectrum of the cured thin film monomer was obtained and the height peaks of both aliphatic and aromatic C=C were measured and recorded using software.

The cross-linking density (CLD) was calculated according to the following formula (Barszczewska-Rybarek et al., 2000):

$$\boldsymbol{CLD} = \frac{2\,\mathrm{DC} - 1}{\mathrm{DC}}$$

where;

DC is the degree of conversion.

Figure 3.9 Digital caliper showing thickness of the thin film used for FT-IR

3.3.4 Determination of volumetric polymerization shrinkage

The volumetric polymerization shrinkage for each experimental resin was determined according to the Archimedes' principle (Uhl et al., 2005; Lohbauer et al., 2009), which is based on the density measurements of both uncured and cured materials. An electronic densitometer (SD-200L, Japan) (Figure 3.10), was used to measure the density of both the uncured and cured resin.

A glass slide was weighed in the air (m_{ga}) and in water (m_{gw}). Then a small amount of uncured resin was placed on the glass slide and weighed in the air (m_{mga}) and in water (m_{mgw}). This procedure was carried out under yellow light to avoid premature polymerization.

The mass of uncured resin (air m_a and in water m_w) were calculated using the following equations (Uhl et al., 2005):

$$\boldsymbol{m_a} = m_{mga} - m_{ga}$$

$$\boldsymbol{m_w} = m_{mgw} - m_{gw}$$

Where;

m_{ga} is the mass weight of glass slide in air

m_{gw} is the mass weight of glass slide in water

m_{mga} is the mass weight of glass slide and uncured resin in air

m_{mgw} is the mass weight of glass slide and uncured resin in water

For each uncured resin, the distilled water temperature was recorded using a thermometer.

The density of uncured resin was then calculated according to the following equation:

$$\boldsymbol{Density\ of\ uncured\ resin} = \left(\frac{m_{\mathrm{a}}}{m_{\mathrm{a}} - m_{\mathrm{w}}}\right) \times \text{density of distilled water}$$

The density of distilled water used in the equation was tabulated in Appendix III, based on the recorded temperature.

The specimens fabricated for 3-point bending test was also used for the determination of the density of cured resins. The specimens were bar-shaped, 2mm in height, 2mm in thickness, and 25 mm in length. After one hour of irradiation, the specimens were weighed in the air (m_{a}) and (m_{w}) in water using an electronic densitometer. The density of cured resin was then calculated using the same equation of density of uncured resin. This procedure was done with extreme care to ensure that no air bubbles adhered on the specimen, otherwise, the specimen was removed from the water and dried using absorbent tissue, and weigh again. Then, the percentage of volumetric polymerization shrinkage (*% VPS*) was calculated using the following equation (Lohbauer et al., 2009):

$$\%\,\boldsymbol{VPS} = \left(\frac{\text{Density of cured resin} - \text{Density of uncured resin}}{\text{Density of cured resin}}\right) \times 100$$

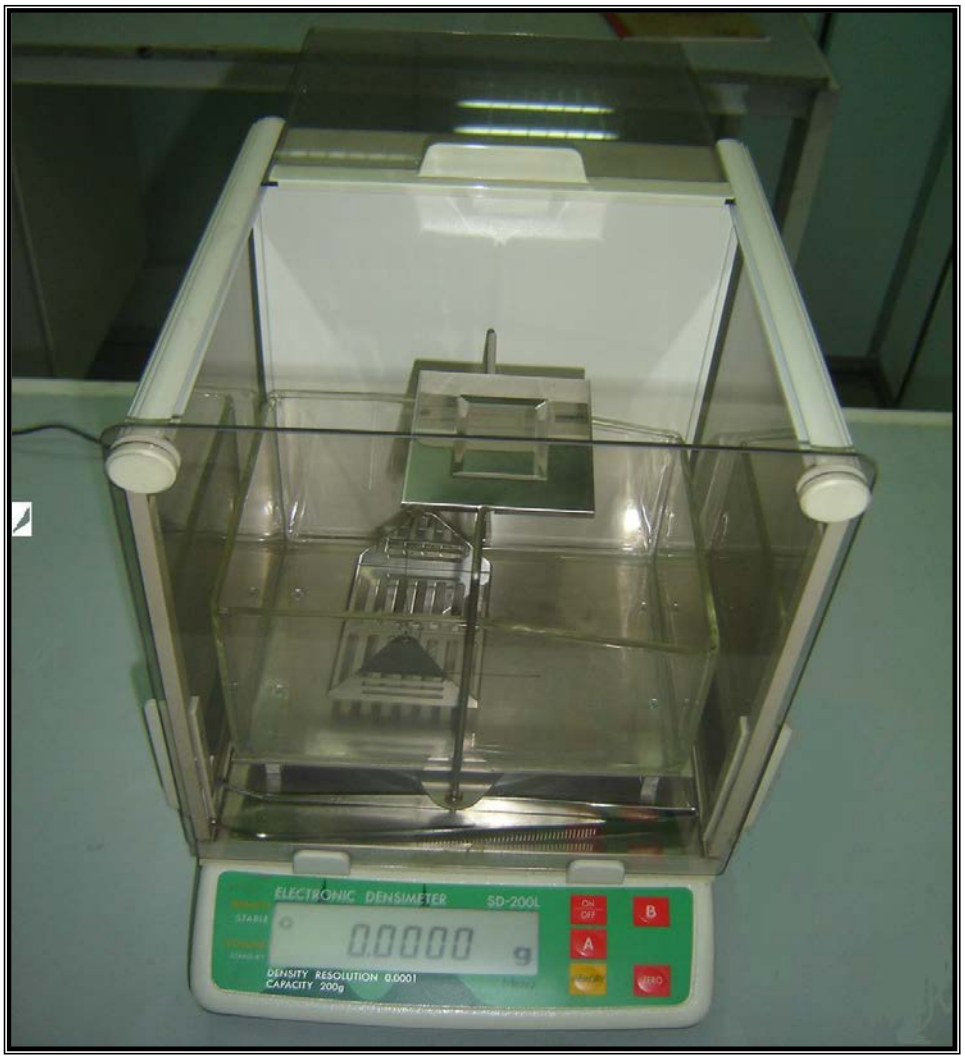


Figure 3.10 Electronic densitometer

3.3.5 Determination of water sorption and solubility

For each experimental resin, 10 disc-shaped specimens (15± 0.1 mm in diameter; and 1.0 ± 0.1 mm in thickness) were fabricated using a plastic mould (Figure 3.11), according to ISO 4049:2000.

The mould was filled with the resin and polyester film was then placed on the mould and covered with a glass slide and finger pressure was applied gently to extrude the excess materials.

The tip of the light curing unit was placed at the center of the specimen and against the glass slide and irradiated for 40 seconds. The tip of the light curing unit was moved to another section overlapping the previous section and irradiated further for 40 seconds. The specimen was irradiated on both sides. The intensity output of the light curing unit was not less than the 500 mW/cm^2, and monitored periodically, where the intensity was measured after every 3 specimens were prepared, throughout the specimens' preparation.

After the specimens were removed from the mould, the irregularities and flash were removed by grinding the periphery of the specimen against silicon carbide paper (1000–1200 grits). The debris was then blown away using compressed air. The volume, V (m^3), for each specimen was calculated from the mean diameter of two measurements at right angles to each other using a digital caliper, and the same was done for the thickness, where five measurements: one in the center and others at points equally spaced on the circumference were made.

The specimens were transferred to a desiccator containing freshly dried silica gel, which were then stored in an oven at 37 ± 1°C for 22 hours. Then the specimens were stored in another desiccator; maintained at 23 ±1°C for a further 2 hours. The specimens were then weighed, using an analytical balance to an accuracy of ±0.1 mg (Acculab,

Sartorious group, Germany). This procedure was repeated until a constant mass (m_1) was obtained, ±0.1 mg within 24 hour period.

All the specimens were immersed in distilled water and stored in an oven at 37 ±1°C for 7 days, before they were removed and washed with water. Surface water was blotted away until the specimens were free from visible moisture, waved in the air for 15 seconds, and weighed 1 min after removal from the water. This mass was recorded as m_2. The specimens were then reconditioned to constant mass in the desiccator containing silica gel using the same procedure. The constant mass was recorded as m_3.

The values for water sorption and solubility were calculated, in micrograms per cubic millimeter (μg/mm^3), for each specimen using the following equations:

$$\textbf{Water sorption} = \frac{m_2 - m_3}{\mathrm{V}}$$

$$\textbf{Water solubility} = \frac{m_1 - m_3}{\mathrm{V}}$$

where;

m_2 is the mass of the specimen, in micrograms, after immersion in water for 7 days;

m_3 is the reconditioned mass of the specimen, in micrograms;

m_1 is the conditioned mass, in micrograms, prior to immersion in water; and

V is the volume of the specimen, in cubic millimeters.

Figure 3.11 Water sorption mould

3.3.6 Determination of flexural strength, modulus of elasticity and toughness

For each experimental resin, 10 bar shaped specimens (25±1 mm in length, 2±0.1 mm thickness, and 2±0.1 mm in height) were fabricated using a split stainless steel mould (Figure 3.12), according to ISO 4049:2000. The split type mould was designed to minimize stress during the specimens' removal from the mould.

Firstly, the metal mould was placed over a glass slide and a polyester sheet. After the resin was packed into the mould, a celluloid strip was put on top of the mould and covered with another glass slide. Then, finger pressure was applied to extrude excess materials from the mould. The tip of the light curing unit was placed at the center of the specimen and against the glass slide and irradiated for 40 seconds. The tip of the light curing unit was moved to another section overlapping the previous section and also irradiated for 40 seconds. The specimen was irradiated on both sides, to assure complete polymerization (Dewaele et al., 2006). The specimens were removed carefully from the mould. Any flash was carefully removed by gently hand-polishing the edges of specimens using a grit silicon carbide paper.

The specimens were then stored in distilled water at 37±1°C for 24 hours. Then the specimens were removed and dried. The dimensions of the specimens were measured to an accuracy of ± 0.01 mm, using a digital caliper. Finally, the specimens were transferred for the flexural strength testing using a high precision Universal Testing machine (Shimadzu Corporation, Japan). The 3-point bending test set up consists essentially of two rods (2 mm in diameter) mounted parallel with a 20 mm space in between, and a third rod (2 mm in diameter) centered between, and parallel to, the other two rods, so that the three rods give a three-point loading to the specimen (Figure 3.13). The load was applied to the specimen at a cross-head speed of 0.75 mm/min until the specimen fractures.

Then the flexural strength and modulus of elasticity were calculated, in pascals according to the following equations:

$$\boldsymbol{Flexural\ strength} = \frac{3Fl}{2bh^2}$$

$$\boldsymbol{Modulus\ of\ elasticity} = \frac{Sl^3}{4bdh^3}$$

where;

F is the maximum load, in Newtons;

l is the support span, in millimeters;

b is the width of specimen, in millimeters;

h is the height of specimen, in millimeters;

S is the load (N), between two convenient points, in the straight portion of the trace.

The toughness or work of fracture, in kilo-joules per each meter square (kJ/m²) was calculated according to the work of fracture equation (Eick et al., 2007; Tian et al., 2007):

$$\boldsymbol{Toughness} = \frac{A}{(bh)}$$

where;

A is the area under the load–deformation curve (which is the work done by the applied load to deflect and fracture the specimen);

b is the width of specimen, in millimeters;

h is the height of specimen, in millimeters.

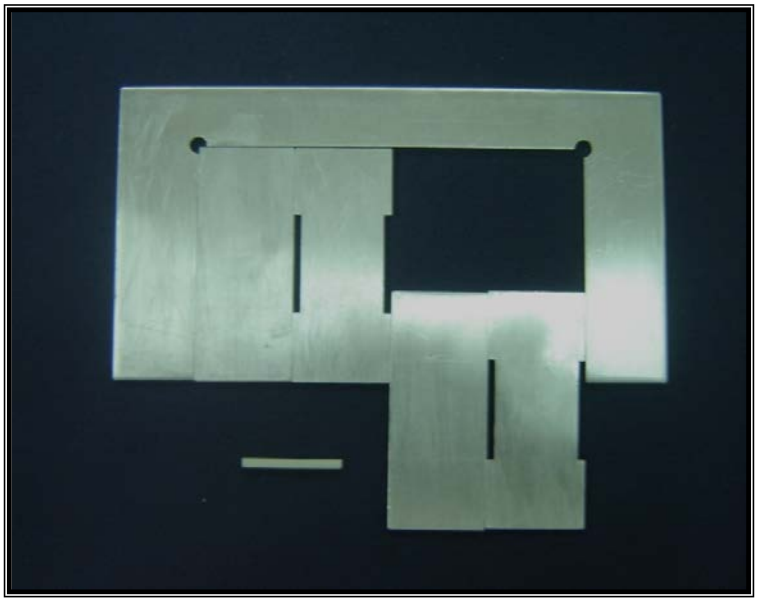


Figure 3.12 Split stainless steel mould

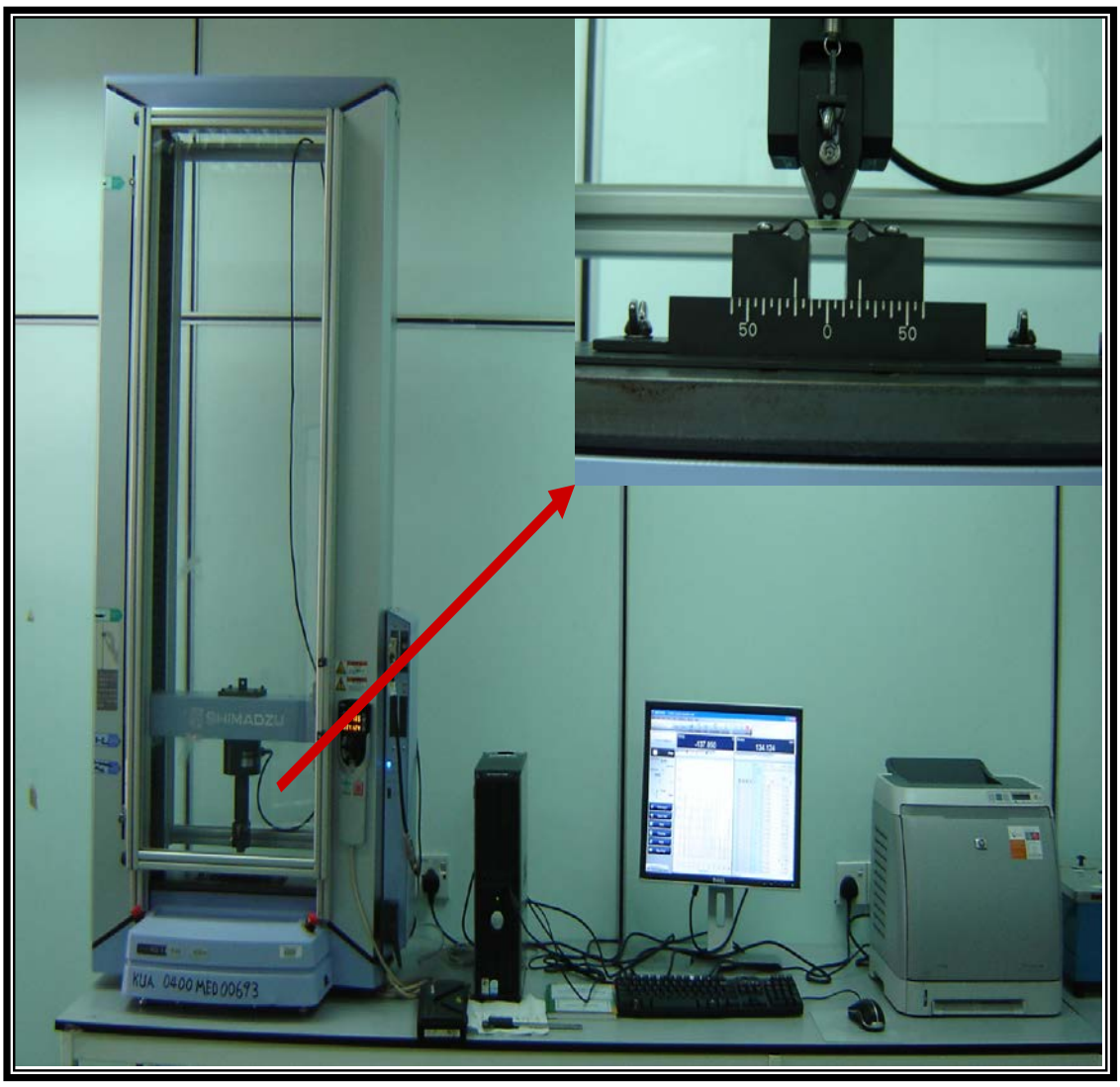


Figure 3.13 3-point bending test using universal testing machine

3.3.6 Data analysis

The descriptive data and statistical test will be carried out to compare between both experimental resins (Bis-GMA and UAM), at p = .05 using SPSS, version 12. All data will be subjected to the normality assumption evaluation, both graphically and statistically. The values of skewness and kurtosis will be also taken into consideration. The acceptable range for these values will be -2 to +2 as described by Tabachnick & Fidell (2001). When data is found to be normally distributed, t-test will be employed for comparison. However, the non-parametric, Mann-Whitney U test will be selected if the data is not normal or the assumption of the outcome is not met.

3.4 Experimental resin systems

3.4.1 Preparation of light-curable resin systems

Five experimental resin systems were prepared, where the diluents monomer TEGDMA and Bis-EMA, coded by T and E respectively, were used as co-monomers with UAM (U) and Bis-GMA (B). The composition (by weight %) of each monomer is illustrated in Table 3.2.

Table 3.2 Composition (percentage by weight) of light-curable resin systems

Experimental resin systems (code)	**Monomers**				**Light-initiators**	
	Bis-GMA	UAM-M	Bis-EMA	TEGDMA	CQ	4EDMAB
BT	74.25%			24.75%	0.2%	0.8%
U/BT	37.12%	49.5%		12.38	0.2%	0.8%
U/E(3/1)		74.25%	24.75%		0.2%	0.8%
U/E(1/1)		49.5%	49.5%		0.2%	0.8%
U/E/BT	24.75%	33%	33%	8.25%	0.2%	0.8%

For each resin system, the percentage monomers were calculated, weighed and mixed. Then, the same procedures for light-curable resin preparation including the filling of resin systems into syringes were carried out as described in Section 3.3.1.

3.4.2 Determination of viscosity

The viscosity for each uncured experimental resin system was determined in accordance to the procedures described earlier in the experimental resin in Section 3.3.2.

3.4.3 Determination of degree of conversion and cross-linking density

For each experimental resin system, the degree of conversion and cross-linking density were determined in accordance to the procedures described earlier in Section 3.3.3.

3.4.4 Determination of volumetric polymerization shrinkage

For each experimental resin system, the volumetric polymerization shrinkage was determined in accordance to the procedure described earlier in Section 3.3.4.

3.4.5 Determination of water sorption and water solubility

For each experimental resin system, the water sorption and water solubility were determined in accordance to the procedures described earlier in Section 3.3.5.

3.4.6 Determination of flexural strength, modulus of elasticity and toughness

For each experimental resin system, the flexural strength, modulus of elasticity and toughness were determined in accordance to the procedures described earlier in Section 3.3.6.

3.4.7 Data analysis

The descriptive analysis and One-way ANOVA will be carried out to ascertain the significant difference amongst the experimental resin systems and further multiple post hoc comparisons will be selected to reveal the existence of significant difference amongst them, where the Bis-GMA/TEGDMA (BT) resin system will be used as a control (Cont) group. However, if the data does not meet the normal distribution assumption, non-parametric statistical tests will be employed.

3.5 Flowable composites

The flowable composite (FC) consists of four freshly prepared experimental FCs and one commercial FC, Esthet.X flow (Dentsply Caulk, USA).

3.5.1 Preparation of experimental flowable composites

For each experimental FC, the monomers and light-initiators were calculated, weighed and mixed first (Table 3.3). Once the monomers and light-initiators produced a clear homogenous mixture, silanated barium borosilicate glass fillers (60% weight) was added and mixed gradually. The average filler size is 0.7 μm with filler of 0.5 μm (10%) and 1.4 μm (90%). Details of the filler used are described in Appendix IV. Then the experimental FCs were filled into syringes and vacuum as described in Section 3.1.1.

Table 3.3 Experimental flowable composites composition (percentage by weight)

Experimental FCs (code)	**Monomers**				**Light-initiators**		**Filler**
	Bis-GMA	UAM	Bis-EMA	TEGDMA	CQ	4EDMAB	Glass
FC-BT	29.25%			9.75%	0.2%	0.8%	60%
FC-U/BT	14.6%	19.5%		4.9%	0.2%	0.8%	60%
FC-U/E		19.5%	19.5%		0.2%	0.8%	60%
FC-U/E/BT	9.75%	13%	13%	3.25%	0.2%	0.8%	60%

3.5.2 Determination of volumetric polymerization shrinkage

For each experimental FC and Esthet.X flow FC, the volumetric polymerization shrinkage was determined following the procedure described earlier in Section 3.3.4.

3.5.3 Determination of volumetric change

For each experimental FC and Esthet.X flow FC, 10 disc specimens were fabricated, using a stainless steel split mould (8±0.1 mm in diameter and 2±0.1mm in height). The mould was held by a slotted plastic sleeve as is shown in Figure 3.14.

The flowable composite material was packed into the mould and was placed over a celluloid strip and held by a plastic block. Again, celluloid strip, glass slide and finger pressure was applied to extrude the excess materials from the mould.

The light curing tip measuring 8 mm in diameter was positioned in the center of the specimen and against the glass slide and the specimen was irradiated for 40 seconds. The specimen was then further irradiated for 40 seconds on the other side. The irradiation time for Esthet.X flow flowable composite was 20 seconds for each side as suggested by the manufacturer. The output of the curing device was monitored to ensure an intensity of not less than 500 mW/cm^2 throughout the procedure. The irregularities and flash were removed by sanding the specimen against a silicon carbide paper (1000–1200 grits). The debris was blown away using compressed air. After one hour of post-curing, the mass in the air (m_a) and in water (m_w) of the specimen were determined by a densitometer.

The specimens were immersed in distilled water and stored in an oven at 37 ±1°C for 7 days. After that, the specimens were removed and washed with water. Surface water was blotted away until free from visible moisture and waved in the air for 15 seconds.

The mass in the air (m_{a7}) and in water (m_{w7}) were then recorded again. Then the volumetric changes were determined using the Archimedes principle. The change in buoyancy of a material in an Archimedean fluid depends on the volumetric changes of the material (Watts et al., 2000; Ruttermann et al., 2007). Volumetric changes percentage (%) were calculated from the changes of densities after the curing and storage period in water, using the following equation:

$$\boldsymbol{Volumetric\ Change}\ (\%) = \left(\frac{m_{a7} - m_{w7}}{m_a - m_w} - 1 \right) \times 100$$

where;

m_a is the mass weight of the specimen in the air after curing

m_w is the mass weight of the specimen in water after curing

m_{a7} is the mass weight of the specimen in the air after 7 days water storage

m_{w7} is the mass weight of the specimen in water after 7 days water storage

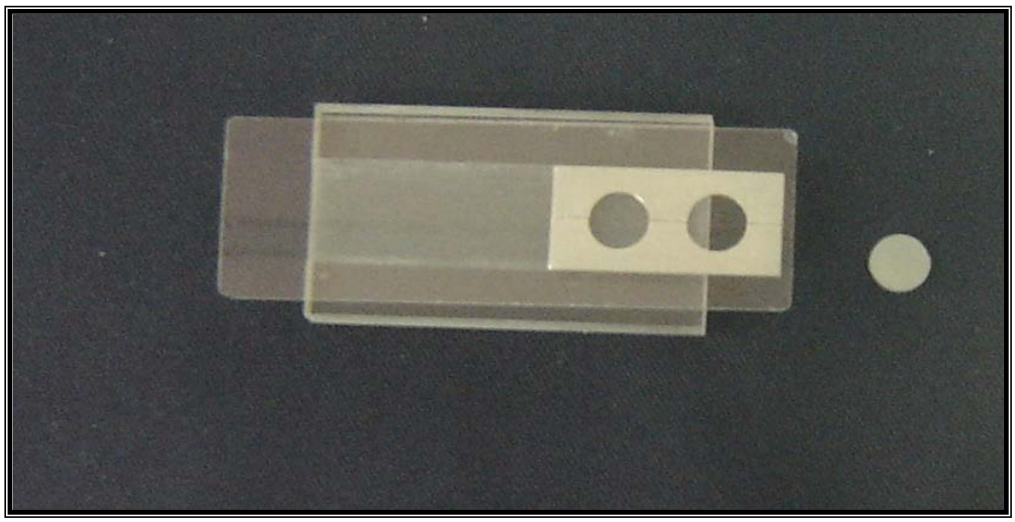


Figure 3.14 Split mould held by a slotted plastic sleeve

3.5.4 Determination of water sorption and water solubility

For each FC, 10 disc specimens (8±0.1 mm in diameter and 2±0.1 mm in height) were fabricated as described earlier in Section 3.5.3. Prior to the specimen immersion in water for 7 days, the volume of the specimens was calculated based on diameter and thickness measurements as described earlier in Section 2.2.5, and the mass of the specimen was recorded as m_0, with no conditioning in desiccators containing silica gel. After water storage, the specimens mass weight was recorded again as m_7. The specimens were then transferred to a desiccator, which contained silica gel, and then stored in an oven at 37 ± 1 °C for 22 hours. After this, the specimens were stored in desiccators maintained at (23±1°C) for 2 hours. This procedure was repeated until the desorbed constant mass (m_{7d}), was obtained to the nearest ± 0.1 mg. The specimens were weighed using an analytical balance with an accuracy of ±0.1 mg.

The water sorption and solubility were then calculated using the followings equations:

$$\textbf{Water sorption} = \frac{m_7 - m_{7\mathrm{d}}}{\mathrm{V}}$$

$$\textbf{Water solubility} = \frac{m_0 - m_{7\mathrm{d}}}{\mathrm{V}}$$

where;

m_7 is the mass weight of the specimen, in micrograms, after immersion in water for 7 days;

m_{7d} is the mass weight of the specimen, in micrograms, after desiccating;

m_0 is the mass weight of the specimen, in micrograms, prior to immersion in water; and

V is the volume of the specimen, in cubic millimeters.

3.5.5 Determination of flexural strength, modulus of elasticity and toughness

For each experimental flowable composite and Esthet.X flow flowable composite, the flexural strength, modulus of elasticity and toughness were determined in accordance with the procedure describe earlier in Section 3.3.6.

3.5.6 Assessment of cytotoxicity

3.5.6.1 Preparation of extract

Ten disc specimens were fabricated for each experimental FC and Esthet.X flow FC, following the procedure described earlier in Section 3.5.3. Before the extraction procedure, the specimens were sterilized in 70% ethanol for 5 minutes, washed twice with sterile phosphate buffered saline (PBS) (GIBCO, Germany), and dried under UV light in a biohazard lamina flow cabinet. Then the extractions of specimens were carried out according to the guidelines described in test procedure ISO 10993-12:1999.

The specimens were immersed in culture medium-Dulbecco's modified Eagle's medium (DMEM) (Sigma D5648, Sigma-Aldrich, St. Louis,MO,USA) and the ratio of the specimen surface area to the volume of the solution (DMEM) was 3 cm^2/ml. The specimens with culture medium (DMEM) were then stored at 37°C in 5% carbon dioxide incubator (Figure 3.15A) for 72 hours. After the incubation period, the extracts were immediately used for the cytotoxicity test. The culture medium without the extraction of specimen was used as a solvent.

3.5.6.2 Cell culture

Mouse fibroblast cell lines, L-929 was purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in 75 cm^2 flasks (Nunc^{TM}, Denmark) (Figure 3.15B), which contained the complete culture medium; DMEM supplemented with 10% fetal calf serum (PAA, GmbH, Germany), and antibiotic-antimycotic (PAA, GmbH, Germany) and stored in a 5% carbon dioxide incubator at 37^{o}C. The culture medium was changed every third day. The subculture monolayer cell was washed twice with PBS. 2 ml of trypsin (GIBCO, Germany) and was added to detach the cells. The cells were then incubated for a further 5 min at 37^{o}C, after which 7~10 ml of fresh complete culture medium was added. The number of cells per milliliter was counted using a hematocytometer, where the concentration of cells was adjusted to 100.000 cell/ml. The flask of cultured cells was then returned to the incubator for 24 hours after which the cells will be ready for seeding using the 96-well plate method for cytoxicity test.

3.5.6.3 Cytotoxic test

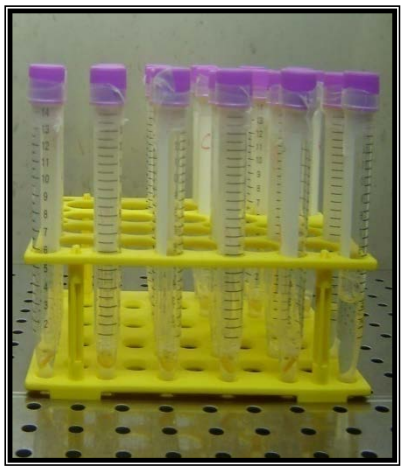
The cytotoxicity of all FCs specimens' extract on L-929 cells was determined by using the 96-well plate (Nunc^{TM}, Denmark) method. A 100 µl aliquot of cell suspension containing 1 X 10^5 cells was placed into each well and incubated for 24 hours to allow cell attachment to the bottom of the well. Then the cultured medium in each well was removed and replaced with extracts.

After 72 hours, 200 µl of Cell Titer 96$^{®}$ A_{queous} One Solution Cell Proliferation reagent (Promega.com, USA) was added to each well and further incubated for 2 hours. Three 96-well plates were used, where the inner wells were used for this study. In the same plate three wells were used to test the extraction of each type of flowable composite. The wells with the cultured medium only were used as solvent control to calculate the

percentage of viable cells. The amount of formazan produced by viable cells was determined by a microplate reader (Multiskan EX spectrophotometer) as is shown in Figure 3.15C. At the end of the experiment the number of viable cells was determined colorimetrically by using the Cell Titer 96® A_{queous} One Solution Cell Proliferation Assay. The assay composed of the tetrazolium compound MTS and the electron coupling reagent, PMS. MTS (yellow) is reduced metabolically in the mitochondria of viable cells to formazan (purple). The details of Cell Titer 96® A_{queous} One Solution Cell Proliferation reagent composition and properties are described in Appendix V. The absorbance can be measured between 450 nm to 540nm. The plates were shaken before the optical densities were measured.

After the optical density (OD) for each well was recorded, the percentage of viable cells in each well was calculated as follows:

$$\%\,\boldsymbol{Viable\ cells} = \frac{OD\ of\ tested\ well}{OD\ of\ control\ well\ (Solvent)} \times 100$$



(A)

(B)

(C)

Figure 3.15 Cytotoxicity test procedure

(A) Specimen extraction
(B) Cell culture in 75cm^2 flasks
(C) Microplate reader

3.5.7 Data analysis

For each FC, the descriptive analysis will be carried out and One-way ANOVA will determine the significance difference between the FCs and further multiple post hoc comparisons will be selected to reveal the existence of significant differences amongst the FCs. The experimental FC-BT which is based on the common Bis-GMA/TEGDMA will be used as the experimental control (Exp-Cont) group and Esthet.X flow will be used as the commercial control (Com-Cont) group. However, if the data does not meet the normal distribution, the non-parametric statistical tests will be employed.