

**MATERIALS AND
EXPERIMENTAL TECHNIQUES**

- 2.1 *Materials*
- 2.2 *Experimental techniques*
- 2.3 *Tests on vulcanizates*

This chapter deals with the details of materials used and the experimental techniques adopted. The various tests done on the vulcanizates prepared for the investigations are also described.

2.1 Materials**2.1.1 Natural rubber (NR)**

The natural rubber used in this study was ISNR-5 of Mooney viscosity (ML (1 + 4) 100 °C) 85, obtained from the Rubber Research Institute of India, Kottayam. The current ISO specifications for the grade of rubber are given in the Table 2.1 [1].

Table 2.1 Specifications for ISNR-5 (ISO 2000: 1989 (E))

Characteristics	Limit	Test method
Dirt content, % (m/m) retained on 45 µm sieve, max.	0.05	ISO 249
Ash, % (m/m), max.	0.60	ISO 247
Initial plasticity, min.	30	ISO 2007
Plasticity retention index (PRI), min.	60	ISO 2930
Nitrogen content, % (m/m), max.	0.60	ISO 1656
Volatile matter content, % (m/m), max.	0.80	ISO 248 (Oven method at 100 ± 5 °C)

Rubber from the same lot has been used for the experiment since it is known that the molecular weight, molecular weight distribution and non-rubber constituents of natural rubber are affected by clonal variation, season, use of yield stimulants and method of preparation [2].

2.1.2 Epoxidised natural rubber (ENR 25)

Epoxidised natural rubber (ENR 25) containing 25 mole percent of oxirane rings, obtained from the Rubber Research Institute of India, Kottayam has been used in the study.

2.1.3 Vulcanizing agent - Sulphur

Sulphur with the specifications shown below was supplied by Standard chemicals Co. Pvt. Ltd., Chennai.

Parameter	Value
Specific gravity	2.05
Acidity, max (%)	0.01
Solubility in CS ₂ (%)	98

2.1.4 Accelerators

2.1.4.1 N-oxydiethylene 2-benzothiazolesulfenamide (MBS)

MBS was obtained from Merchem Ltd., Cochin, India. The specification of MBS is shown below.

Parameter	Value
Melting point (°C)	Initial 80
	Final 83
Density (25 °C, kg/m ³)	1400

2.1.4.2 Tertiarybutyl benzothiazolesulfenamide (TBBS)

TBBS was obtained from Merchem Ltd., Cochin, India. The specification of TBBS is as shown below.

Parameter		Value
Melting point (°C)	Initial	105
	Final	107
Density (25 °C, kg/m ³)		1300

2.1.4.3 N, N-dicyclohexyl-2-benzothiazolesulfenamide (DCBS)

DCBS was obtained from Merchem Ltd., Cochin, India. The specification of DCBS is shown below.

Parameter		Value
Melting point (°C)	Initial	99
	Final	101
Density (25 °C, kg/m ³)		1250

2.1.4.4 N, N-dibenzyl-2-benzothiazolesulfenamide (DBBS)

DBBS was obtained from Merchem Ltd., Cochin, India. The specification of DBBS is:

Parameter		Value
Melting point (°C)	Initial	92
	Final	94
Density (25 °C, kg/m ³)		1290

2.1.4.5 Tetramethyl thiuramdisulfide (TMTD)

TMTD was obtained from Merchem Ltd., Cochin, India. The specification of TMTD is as shown below.

Parameter		Value
Melting point (°C)	Initial	144
	Final	148
Density (25 °C, kg/m ³)		1450

2.1.4.6 Tetrabenzyl thiuramdisulfide (TBzTD)

TBzTD was obtained from Merchem Ltd., Cochin, India. The specification of TBzTD is:

Parameter		Value
Melting point (°C)	Initial	126
	Final	130
Density (25 °C, kg/m ³)		1450

2.1.5 Fillers

2.1.5.1 High abrasion furnace black (HAF-black)

High abrasion furnace black (N330) used in the study was supplied by M/s Philips carbon black India Ltd., Cochin, India. The specifications of the black are:

Parameter	Value
Appearance	Black granules
DBP absorption (cc/100g)	102 ± 5
Pour density (kg/m ³)	376
Iodine adsorption number (mg/g)	82

2.1.5.2 Precipitated silica

Precipitated silica of commercial grade supplied by Minar Chemicals, Cochin. The following are the specifications.

Parameter	Value
pH (5 % aqueous solution)	6.3
Density (g/cc)	2.03
SiO ₂ content (%)	90

2.1.6 Antioxidants

2.1.6.1 N-(1, 3-dimethylbutyl)-N²-phenyl-p-phenylenediamine (6PPD)

6PPD was obtained from Merchem Ltd., Cochin, India. The specifications of the 6PPD are:

Parameter	Value	
Melting point (°C)	Initial	46
	Final	49
Density (25 °C, kg/m ³)	1090	

2.1.6.2 Polymerized 1, 2-dihydro-2,2,4-trimethyl quinoline (TQ)

TQ was obtained from Merchem Ltd., Cochin, India. The specification of TQ is given below.

Parameter	Value
Softening point (°C)	83-97
Dimer content (%)	45
Bis-aniline content (%)	0.2
Density (25 °C, kg/m ³)	1100

2.1.6.3 High pure grade TQ (HPG)

HPG is polymerized 1,2-dihydro-2,2,4-trimethyl quinoline with dimer as the predominant constituent, obtained from Merchem Ltd., Cochin, India. The specification of HPG is shown below.

Parameter	Value
Softening point (°C)	83-100
Dimer + Trimer content (%)	70
Bis-aniline content (%)	0.1
Density (25 °C, kg/m ³)	1100

2.1.7 Other chemicals

2.1.7.1 Zinc oxide (ZnO)

Zinc oxide was supplied by M/s Meta Zinc Ltd., Mumbai. The specifications are:

Parameter	Value
Specific gravity	5.5
ZnO content (%)	98
Acidity (%)	0.4

2.1.7.2 Stearic acid

Stearic acid was supplied by M/s Godrej Soaps Pvt. Ltd., Mumbai. The specifications are as shown below.

Parameter	Value
Specific gravity	0.85
Melting point (°C)	50-69
Acid number	185-210

2.1.7.3 Naphthenic oil

M/s. Hindustan Petroleum Ltd., India, supplied naphthenic oil. The specifications are:

Parameter	Value
Aniline point (°C)	78
Viscosity gravity constant	0.85-0.9
Aromaticity (%)	20

2.1.7.4 Diethylene glycol (DEG)

Diethylene glycol was supplied by M/s Merck Limited, Navi Mumbai, India. The specifications are as shown below.

Parameter	Value
Boiling point (°C)	245
Flash point (°C)	140
Density (at 20 °C)	1.12
Viscosity (at 40 °C, cSt)	43

2.1.7.5 Coupling agent

The coupling agent used was Si69, a product of Degussa AG, Germany. Chemically the product is bis(triethoxysilylpropyl) tetrasulfide. The specifications of the product are shown below [3].

Parameter	Value
Sulphur content (%)	22.7
Volatiles (%)	< 4.0
Average molecular weight (g/mol)	532
Density (g/cc)	2.5
Appearance	Clear yellow liquid

2.1.7.6 Toluene

Toluene (specifications shown below) was supplied by M/s. Fine chemicals Ltd., Mumbai.

Parameter	Value
Boiling point (°C)	110
Appearance	Colourless liquid
Odour	Aromatic

2.1.7.7 Diesel oil

Diesel oil (specifications shown below) was of commercial grade purchased from Bharat Petroleum Company Ltd. (BPCL).

Parameter	Value
Boiling point (°C)	>110
Flash point (°C)	>35
Density (g/cc)	0.82 - 0.86
Viscosity (kinematic at 40 °C, cSt)	2-5

2.1.7.8 Lube oil

Lube oil (specifications shown below) was of commercial grade supplied by Castrol.

Parameter	Value
Boiling point (°C)	>250
Flash point (°C)	>180
Density (g/cc)	<1
Viscosity (kinematic at 40 °C, cSt)	32

2.2 Experimental techniques

2.2.1 Mixing and homogenization of the NR compounds

The natural rubber compounds were prepared on a laboratory size two-roll mixing mill as per ASTM D 3182 [4] at a friction ratio of 1:1.25.

The compounding ingredients were added as per the procedure given in ASTM D 3184 [5].

2.2.2 Cure characteristics

The rheographs of the mixes and their cure characteristics were obtained using RPA 2000 Rubber Processing Analyzer (Alpha Technologies, model - 90AJC2353) as per ASTM D 5289 [6]. The instrument used has two directly heated opposing biconical dies that are designed to achieve a constant shear gradient over the entire sample chamber. The lower die is oscillated at 50 rpm and the torque transducer on the upper die senses the force being transmitted through the rubber. To determine the cure characteristics of the rubber compound, approximately 5 g was placed in the lower die that is oscillated through a small deformation angle $\pm 0.2^\circ$ at a frequency of 50 cycles per minute. The torque transducer on the upper die senses the forces being transmitted through the rubber. The torque is plotted as a function of time. A typical cure curve is shown in Figure 2.1.

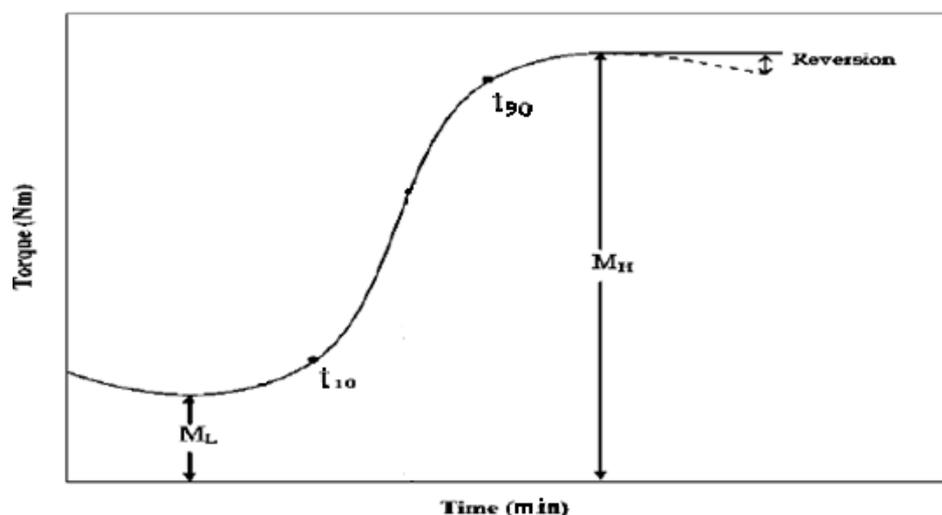


Figure 2.1 Typical cure curve obtained from RPA 2000

The following data can be taken from the cure curve.

- a) Minimum torque (M_L): Measure of the stiffness of unvulcanized test specimen. It is the torque shown by the mix at the test temperature before the onset of cure.
- b) Maximum torque (M_H): Measure of the stiffness or shear modulus of the fully vulcanized test specimen at the vulcanization temperature. It is the torque recorded after curing of the mix is completed.
- c) Scorch time (t_{10}): It is the time taken for attaining 10 % of the maximum torque.
- d) Optimum cure time (t_{90}): Time taken for attaining 90 % of the maximum torque. It is the time corresponding to a torque equal to $0.9(M_H - M_L) + M_L$.
- e) Cure rate index (CRI): It is a measure of the rate of cure and is calculated using the formula,

$$CRI = \frac{100}{(t_{90} - t_{10})} \text{-----} (2.1)$$

2.2.3 Assessment of rubber-filler interactions

2.2.3.1 Strain-sweep analysis

The strain sweep measurements on the uncured and cured samples were conducted to study the rubber-filler interaction. Rubber Process Analyzer (RPA 2000 - Alpha Technologies) is a purposely modified commercial dynamic rheometer for capturing strain and torque signals, with the assistance of appropriate software [7]. Filled rubber compounds exhibit strong nonlinear viscoelastic behaviour, the well-known Payne effect, i.e., the reduction of elastic modulus with increasing strain amplitude [8]. RPA can do strain-sweep tests in which the variation of storage modulus (G'),

loss modulus (G'') and complex modulus (G^*) with change in strain amplitude are measured. With respect to its measuring principle, the RPA cavity must be loaded with a volume excess of test material. According to ASTM D 5289, the manufacturer recommends to load samples of about 5.0 g for a standard filled rubber compound. Samples for RPA testing were consequently prepared by die cutting 46 mm diameter disks out of around 2 mm thick sheets of materials. The testing temperature was selected as 100 °C; a temperature below the curing temperature and the shear strain was varied from 0.7 to 70 % keeping the frequency at 0.5 Hz.

For strain sweep studies of the cured samples, uncured samples were taken and immediately after a cure cycle at their optimum cure time within the RPA cavity, strain sweep at 100 °C was carried out.

2.2.3.2 Bound rubber content

The bound rubber that formed in the master batches can be determined by the following procedure [9]. About 0.2 g of uncured master batch (without curatives) was put into a previously weighed metal cage made by stainless steel wire gauze of 280 mesh and immersed in 20 ml of toluene for 72 hours at room temperature. Toluene was renewed every 24 hours.

The sample was removed from toluene after 72 hours, dried at 105 °C for 24 hours and weighed. The bound rubber content was calculated according to the equation:

$$\text{Bound rubber content (\%)} = \left[\frac{(W_{fg} - W_f)}{W_p} \right] \times 100 \text{ ----- (2.2)}$$

where W_{fg} is the weight of filler with the bound rubber attached, W_f is the weight of filler in the specimen and W_p is the weight of polymer in the specimen.

2.2.4 Moulding

The test specimens were prepared by moulding in an electrically heated hydraulic press at a pressure of 200 kg/cm^2 and at a temperature of $150 \text{ }^\circ\text{C}$ for the optimum cure time. The mouldings were cooled quickly in water at the end of the curing cycle and stored for maturation in a cool dark place.

2.3 Tests on vulcanizates

2.3.1 Mechanical properties

2.3.1.1 Stress-strain properties

The tensile strength, elongation at break and modulus at various elongations were determined using dumb-bell shaped specimens punched out from the vulcanized sheet along the mill grain direction using a dumb bell die (C-type). The tensile properties of the specimens were measured according to ASTM D 412 [10] using Shimadzu Universal Testing Machine, model -AG-1 series (10 KN) at a cross head speed of 500 mm/min .

2.3.1.2 Tear strength

The tear strength test was carried out as per ASTM D 624 [11] using unnicked, 90° angle test pieces. The samples were cut from the compression moulded sheets parallel to the mill grain direction. The measurements were carried out on a Shimadzu Universal testing machine (10 KN) with a grip

separation of 40 mm, using a crosshead speed of 500 mm/min. The tear strength was reported in N/mm.

2.3.1.3 Thermo-oxidative ageing

The thermo-oxidative ageing of the samples were done in a hot air oven at 70 °C and 100 °C for 24 hours according to ASTM D 573 [12] to determine the effect of elevated temperature and time on stress-strain properties. The aged specimens were kept at room temperature for at least 16 hours before the mechanical properties are tested. These are compared with the properties determined on unaged specimens.

2.3.1.4 Hardness

The hardness (Shore A) of the samples was determined using Mitutoyo hardmatic hardness tester according to ASTM D 2240 [13]. The measurement was done by pressing the indenter against the sample and reading the scale, which is ranging from zero (soft) to 100 (hard). The tests were performed on unstressed samples of 12 mm diameter and 6 mm thickness.

2.3.1.5 Compression set

Compression set at constant strain was measured according to Method B of ASTM D 395 [14]. Samples were compressed to constant strain (25 %) and kept for 22 hours in an air oven at 70 °C. At the end of the test period the test specimens were taken out, kept at room temperature for 30 minutes and the final thickness was measured. The compression set in percentage was calculated as:

$$\text{Compression set (\%)} = \frac{(T_0 - T_1)}{(T_0 - T_S)} \times 100 \text{ ----- (2.3)}$$

where T_0 and T_1 are the initial and the final thickness of the specimen and T_S is the thickness of the spacers used.

2.3.1.6 Rebound resilience

Resilience is the energy returned by a vulcanized elastomer when it is suddenly released from a state of strain or deformation. High resilience causes bounce or snap often associated with products of natural rubber. Resilience is defined as the ratio of energy given up on recovery from deformation to the energy required to produce the deformation. Rebound resilience was determined by vertical rebound method according to ASTM D 2632 [15]. Rebound resilience is defined as the ratio of the energy of the metal plunger after impact to its energy before impact expressed as a percentage and hence, in the case where the plunger falls under gravity, is equal to the ratio of rebound height to the drop height.

$$\text{Rebound resilience(\%)} = \frac{\text{Rebound height}}{\text{Drop height}} \times 100 \text{ ----- (2.4)}$$

2.3.1.7 Abrasion resistance

The abrasion resistance of the samples was determined using a DIN Abrader (DIN 53, 516). Samples having a diameter of 6 ± 0.2 mm and a thickness of 12 mm were kept on a rotating sample holder and a 10 N load was applied. Abrasion loss was measured as per ASTM D 5963 [16]. Initially a pre run was given for all samples and its weight was taken. The weight after final run was also noted. The difference in weight is the weight loss by its travel through 42 cm on a standard abrasive surface. The abrasion loss in cm^3/h was calculated using the formula given below:

$$\text{Abrasion loss} = \frac{\text{Loss of weight} \times 60}{\text{Specific gravity} \times 2.2} \text{ ----- (2.5)}$$

Abrasion resistance is the reciprocal of volume loss on abrasion.

2.3.2 Swelling Studies

Vulcanizate samples weighing about 0.3 g were cut using a sharp edged disc shaped die. The samples were immersed in airtight diffusion bottles containing about 20 ml of toluene maintained at constant temperature (25 °C). Samples were removed from the bottles at periodic intervals, the surfaces were quickly dried using tissue paper, weighed and allowed to swell again until equilibrium swelling [17].

2.3.2.1 Transport properties

The mole percent solvent uptake (Q_t) of the sample was calculated from the diffusion data as [18,19]:

$$Q_t(\%) = \frac{\text{Wt.of the solvent absorbed at a given time/Molecular wt.of the solvent}}{\text{Initial wt.of the rubber specimen}} \times 100 \quad \text{----- (2.6)}$$

At equilibrium swelling, Q_t becomes Q_α . The mechanism of diffusion was investigated using the equation [20]:

$$\log (Q_t / Q_\alpha) = \log k + n \log t \quad \text{----- (2.7)}$$

The value of k depends on the structural features of polymer, whereas the value of n determines diffusion mechanism [21]. In the Fickian mode, case 1, the value of n is 0.5 and it occurs when the rate of diffusion of penetrant molecules is much less than the relaxation rate of the polymer chains. In case 2, i.e. non-Fickian transport, where the n value is 1, the diffusion is rapid when compared with the simultaneous relaxation. However in the case of anomalous transport where the n value is in between 0.5 and 1, both solvent diffusion and polymer relaxation rate are

comparable. The effective diffusivity, D of the rubber-solvent system was calculated from the initial portion of the sorption curves using the equation [22, 23]:

$$D = \pi \left(\frac{h\theta}{4Q_\alpha} \right)^2 \text{-----} (2.8)$$

Where h is the initial thickness of rubber sample, θ the slope of the linear portion of the sorption curve Q_t versus $t^{1/2}$. The permeation of a solvent into a polymer membrane will also depend on the sorptivity of the penetrant in the membrane. Hence sorption coefficient (s) has been calculated using the relation [24]

$$s = \frac{W_s}{W} \text{-----} (2.9)$$

where W_s is the weight of the solvent at equilibrium swelling and W , the initial weight of the polymer sample. Since the permeability depends on both diffusivity and sorptivity, the permeation coefficient has been determined using the relation [25]:

$$P = D \times s \text{-----} (2.10)$$

The swollen sample was weighed, solvent removed in vacuum, and the sample was weighed again.

2.3.2.2 Crosslink density

As diffusion is influenced by polymer morphology, the molar mass between crosslinks M_c from the sorption data is also determined. The crosslink densities of the vulcanizates ($1/2M_c$) were determined using the equilibrium swelling data [26]. Degree of swelling usually is expressed as V_r . V_r is dependent on the swelling power of the solvent (high swelling

power means low V_r) and the cross link density. Higher cross link density results in restraint on the network, which results in lower swelling. The volume fraction (V_r) in the swollen network was calculated by the method reported by Ellis and Welding [27] using the following equation:

$$V_r = \frac{(D-FT)\rho_r^{-1}}{(D-FT)\rho_r^{-1} + A_0\rho_s^{-1}} \text{-----(2.11)}$$

where D is the deswollen weight of the test specimen, T is the weight of the test specimen, F is the weight fraction of insoluble components, A_0 is the weight of the solvent absorbed, ρ_r and ρ_s are the densities of the polymer and the solvent respectively. The crosslink density was determined using the Flory-Rehner equation [28]:

$$M_c = \frac{-(\rho_r V_s V_r^{1/3})}{[\ln(1-V_r) + V_r + \chi V_r^2]} \text{----- (2.12)}$$

where V_s is the molar volume of the solvent and the value of the interaction parameter (χ) for NR-toluene taken was 0.42 [29]. The volume fraction of rubber (V_r) is calculated assuming that the filler does not swell. It is then converted to V_{r0} (the value V_r would have in the absence of filler) according to Cunneen and Russel [30].

$$\frac{V_{r0}}{V_r} = ae^{-z} + b \text{-----(2.13)}$$

Here a and b are constants characteristic of the system and z is the weight fraction of the filler in the vulcanizate. The values for a and b for HAF black-filled systems are; a = 0.56 and b = 0.44. The corresponding values for silica-filled [31] systems are a = 1.41 and b = -0.41.

The values of V_{r0} were then substituted in the Flory-Rehner equation in place of V_r to obtain the crosslink density. Using the value M_c , crosslink density (ν) can be calculated using the equation:

$$\nu = \frac{1}{2M_c} \text{-----} \quad (2.14)$$

The swelling index was determined by using the equation:

$$\text{Swelling index} = \frac{(W_2 - W_1)}{W_1} \text{-----} \quad (2.15)$$

where W_1 and W_2 are the weights of the unswollen and swollen rubber, respectively. The swelling index is a direct measurement of degree of cross linking, the smaller the value, the higher the degree of crosslinking.

2.3.2.3 Gibbs free energy change and entropy change

The expansion of the rubber in the presence of a solvent will significantly modify the conformational entropy (ΔS) and elastic Gibbs free energy (ΔG). The elastic Gibbs free energy can be determined from the Flory-Huggins equation [32].

$$\Delta G = RT (\ln (1 - V_r) + V_r + \chi V_r^2) \text{-----} \quad (2.16)$$

From the statistical theory of rubber elasticity, ΔS can be obtained from the relation [33]:

$$\Delta G = -T \Delta S \text{-----} \quad (2.17)$$

which assumes that no change in internal energy of the network occurs upon stretching.

2.3.3 Fluid resistance

The fluid resistance of the vulcanizates was carried out in diesel and lube oil according to the ASTM D 471[34]. Test specimens of circular shape (diameter 20 mm and thickness 2 ± 0.1 mm) were punched out from tensile sheets and initial weight in air was taken to the nearest 1 mg accurately. The test specimens were immersed in oil for 3 days at room temperature. After the immersion time, they were taken out and quickly dipped in acetone, blotted lightly with a filter paper and final weight of the specimens were determined to the nearest 1 mg. The percentage change in mass was calculated as follows:

$$\text{Change in mass(\%)} = \frac{(M_2 - M_1)}{M_1} \times 100 \text{ -----(2.18)}$$

M_1 = initial mass of specimen in air

M_2 = mass of specimen in air after immersion

2.3.4 Thermogravimetric analyses

Thermogravimetric analysis (TGA) is a technique by which the mass of the sample is monitored as a function of temperature or time, while the substance is subjected to a controlled temperature program [35]. Thermogravimetric analysis is used to investigate thermal degradation and thus the thermal stability of the samples.

Thermogravimetric analyses of the samples were carried out in a TGA Q-50 thermal analyzer (TA Instruments) under nitrogen atmosphere. The samples were heated from room temperature to 800 °C at a heating rate of 20 °C/min. The chamber (furnace) was continuously swept with nitrogen at a rate of 40-60 cm³/min. Sample weight varied from 6-10 mg. The weight

changes were noted with the help of an ultra sensitive microbalance. The data of weight loss versus temperature and time was recorded online using the TA Instrument's Q Series Explorer software. The analysis of the thermogravimetric (TG) and derivative thermogravimetric (DTG) curves were done using TA Instrument's Universal Analysis 2000 software version 3.3 B. Thermograms were recorded from room temperature to 800 °C. The onset of degradation temperature (T_i), the temperature at which weight loss is maximum (T_{max}), and weight loss at 500 °C, etc were evaluated.

2.3.5 Scanning electron microscopy (SEM)

SEM was used to investigate the morphology of fractured surfaces [36]. In SEM, the electron beam incident on the specimen surface causes various phenomena of which the emission of secondary electrons is used for the surface analysis. Emitted electron strikes the collector and the resulting current is amplified and used to modulate the brightness of the cathode ray tube. There is one-to-one correspondence between the number of secondary electrons collected from any particular point on the specimen surface and the brightness of the analogous point on the screen and thus an image of the surface is progressively built up on the screen.

In the present study, morphological characterisation of the fractured surfaces of the tensile test specimens was carried out using scanning electron microscope (JEOL Model JSM – 6390 LV) after sputter coating the surface with gold.

2.3.6 Dynamic mechanical analysis

Dynamic mechanical analysis (DMA) was carried out on a DMA Q 800 dynamic mechanical analyzer (TA instruments) using tension film

mode. Samples for DMA measurement were cut from the vulcanized sheets. All samples were tested from 40 to 120 °C at a heating rate of 3 °C/ min at a frequency of 1 Hz. Oscillating strain was set at 0.15 % for all tests.

2.3.7 *In vitro* cytotoxic effect of the NR vulcanizates

Determination of *in vitro* cytotoxic effect of the NR vulcanizates was done in cultured L929 cells as per test procedure based on ISO 10993-5 [37]. L929 (Fibroblast cells) cell lines purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (DMEM - HIMEDIA) which is a medium containing amino acids and nutrients, supplemented with 10 % Fetal Bovine Serum (FBS), which are hormones for cell growth (Invitrogen – brand name). These cells are then grown to confluency at 37 °C in 5 % CO₂ in humidified atmosphere in a CO₂ incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025 % Trypsin in PBS/ 0.5mM EDTA solution (Himedia)) for 2 minutes and transferred to T flasks (flask for cell culture) in complete aseptic conditions. Trypsinization is the process of cell dissociation using trypsin, a proteolytic enzyme which breaks down proteins, to dissociate adherent cells from the vessel in which they are being cultured. PBS is phosphate buffered saline.

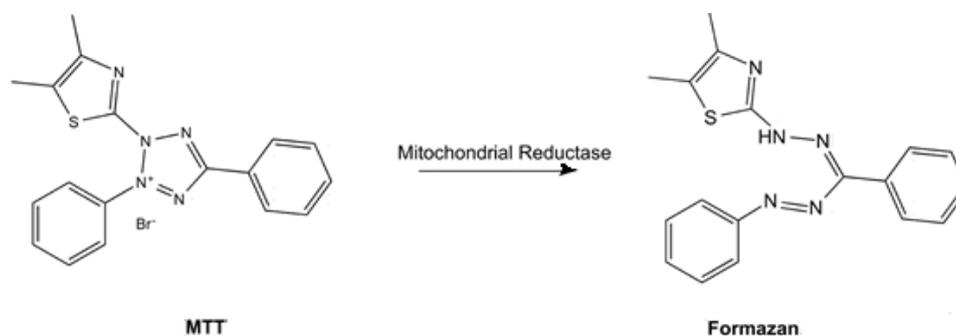
The polymer samples were sterilized in UV chamber for 1 hour. The samples were aseptically transferred to a well plate using sterile forceps. 1 ml of DMEM was added to the plate containing the samples. It was then incubated for 24 hours (one day immersion in medium). The media was removed from the polymer and this medium containing leached polymer was added to 80-90 % confluent cells (kept for 24 hrs). The phase contrast images (under magnification of 20 x) were captured using inverted microscope

(OLYMPUS CKX 41). The cytotoxicity of the polymer samples were determined using MTT assay after 24 hours of incubation.

2.3.7.1 MTT assay

MTT is a colorimetric assay [38] that measures the reduction of yellow 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) by mitochondrial enzyme succinate dehydrogenase.

The cells were washed with 1x PBS and 30 μ l of MTT solution was added to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37 °C for 3 hours. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. MTT reaction in mitochondria of viable cell is as shown in Scheme 2.1 [39].



Scheme 2.1 Formation of formazan from MTT

MTT was removed by washing with 1x PBS and the cells are then solubilised with 200 μ l of an organic solvent (dimethyl sulfoxide (DMSO-Himedia)). Incubation was done at room temperature for 30 minutes until the cell got lysed (broken) and coloured solution was obtained. This solution was transferred to centrifuge tubes and centrifuged at top speed (3500-4000 rpm) for 2 minutes to precipitate cell debris. Optical density of the

solubilized formazan product was read at 540 nm using DMSO as blank in a microplate reader (ELISASCAN, ERBA).

Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. Control samples used for the MTT assay are the cells (L929 cells) to which polymer solution is not added.

$$\% \text{ viability} = \frac{\text{Optical density of test specimen}}{\text{Optical density of control}} \times 100 \text{ -----(2.19)}$$

The material was graded according to a cytotoxicity scale ranging from 0-3 as per the test procedure based on ISO 10993-5 [37]. The grading was done as follows: If the confluency of healthy cells (% viability) is greater than 80 %, it is graded as '0' (non-cytotoxic); if it is between 60-80 %, the grade given is '1' (mildly cytotoxic); if it is 40-60 %, the material is graded as '2' (moderately cytotoxic) and if it is less than 40 %, a grading of '3' (severely cytotoxic) is given [40, 41].

The MTT assay procedure is as represented by the flow diagram (Figure 2.2) shown below:

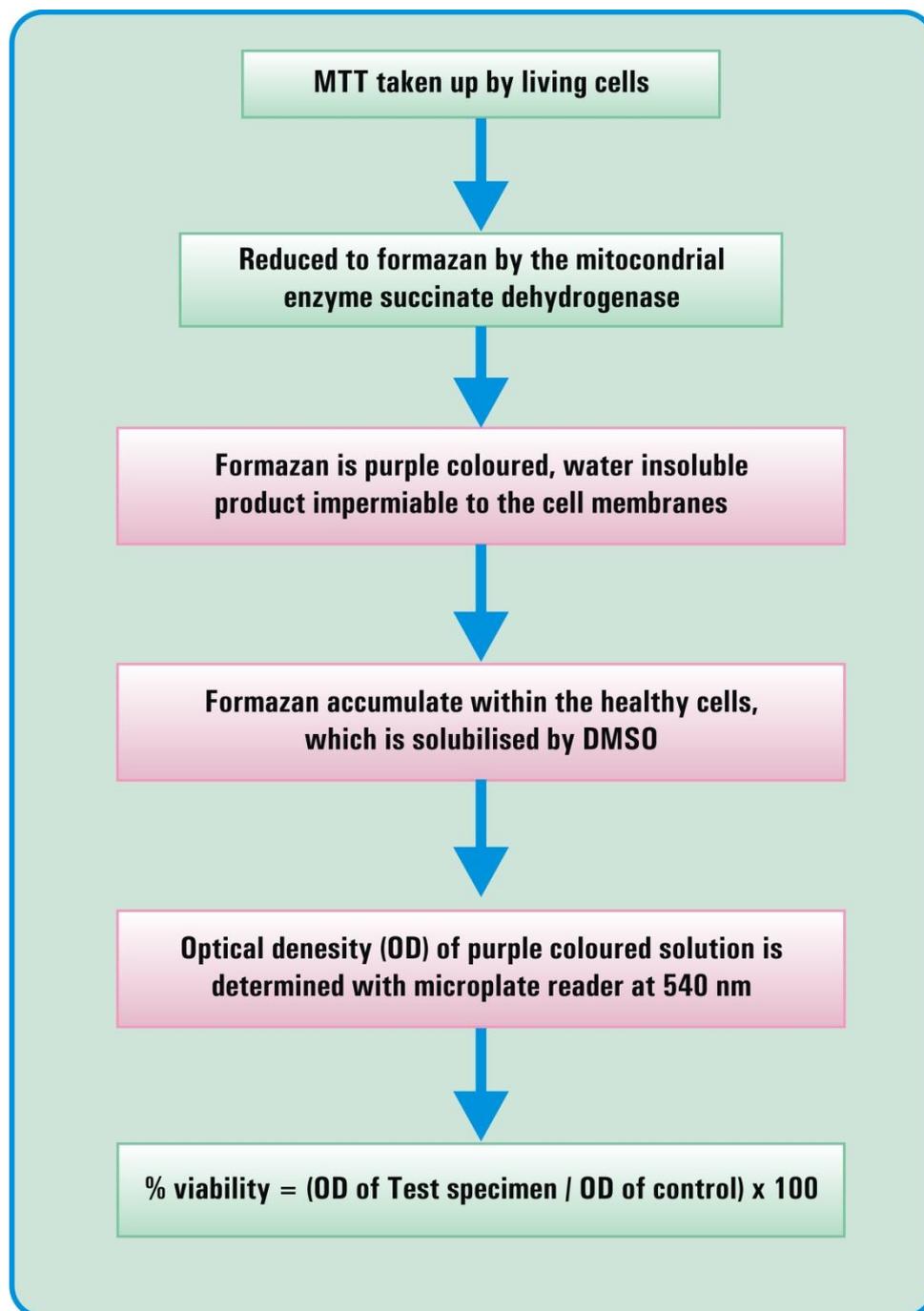


Figure 2.2 Flow diagram for MTT assay procedure

References

- [1] Kurian T. and Mathew N.M., S. Kalia and L. Averous (ed), Biopolymers: Biomedical and Environmental Applications, Wiley-Scrivener, (2011).
- [2] Subramanyam A., Proc. of R. R. I. M. Planter's Conference, Kuala Lumpur, (1971), 255.
- [3] Degussa, Product Information Sheet on Ultrasil VN3, PI 203. IE- from the website www.degussa-fp.com.
- [4] ASTM D 3182, Standard Practice for Rubber - Materials, Equipment, and Procedures for Mixing Standard Compounds and Preparing Standard Vulcanized Sheets.
- [5] ASTM D 3184, Standard Test Methods for Rubber - Evaluation of NR (Natural Rubber).
- [6] ASTM D 5289, Standard Test Method for Rubber Property - Vulcanization Using Rotorless Cure Meters.
- [7] Jean L. L. and Marie C., J. Appl. Polym. Sci., **80**(11), (2001), 2093-2104.
- [8] Payne R. and Whittaker W.E., Rubber Chem. Technol., **44**, (1971), 440.
- [9] Kazumasa Y., Tetsuro O. and Mutsuhisa F., J. Appl. Polym. Science, **85**, (2002), 2053.
- [10] ASTM D 412, Standard Test Methods for Vulcanized Rubber and Thermoplastic Elastomers-Tension.

- [11] ASTM D 624, Standard Test Method for Tear Strength of Conventional Vulcanized Rubber and Thermoplastic Elastomers.
- [12] ASTM D 573, Standard Test Method for Rubber-Deterioration in an Air Oven.
- [13] ASTM D 2240, Standard test method for rubber property-durometer hardness.
- [14] ASTM D 395, Standard Test Methods for Rubber Property-Compression Set.
- [15] ASTM D 2632, Standard Test Method for Rubber Property-Resilience by Vertical Rebound.
- [16] ASTM D 5963, Standard Test Method for Rubber Property-Abrasion Resistance (Rotary Drum Abrader).
- [17] Byran G. and Welding G.W., Techniques of Polymer Science, Society of Chemical Industries, **17**, (1963), 75.
- [18] Mathew G., Singh R. P., Nair N. R. and Thomas S., J. Mater. Sci., **38**, (2003), 2469.
- [19] Sheela J., Kumaran M. G., and Thomas S., Plast. Rubber Compos. Process. Appl., **27**, (1998), 82.
- [20] Chiou J. S. and Paul D. R., Polym. Eng. Sci., **26**, (1986), 1218.
- [21] Lucht L.M. and Peppas N.A., J Appl Polym Sci, **33**, (1987), 1557.
- [22] Crank J., The mathematics of diffusion. Second Ed. Oxford: Clarendon Press, (1975), 244.

- [23] Britton L. N., Ashman R.B., Aminabhavi T. M. and Cassidy P. E, J. Chem. Edn., **65**, (1988),368.
- [24] Aprem A. S., Joseph K., Mathew A. P. and Thomas S., J. Appl. Polym. Sci., **78**, (2000), 94.
- [25] Khinnava R. S. and Aminabhavi T. M., J.Appl.Polym. Sci., **42**, (1991), 2321.
- [26] Gorton A.D.T. and Pendle T.D., Nat. Rubber Technol., **7**, (1976), 77.
- [27] Ellis B. and Welding G.W., Rubb. Chem. Technol., **37**, (1964), 571.
- [28] Flory P.J. and Rhener J., J. Chem. Phys., **11** (1943) 5120.
- [29] Aprem A.S., Joseph K., and Thomas S., J. Applied Polymer Science, **91**, (2004), 1070.
- [30] Cunneen J. I. and Russel R. M., Rubb. Chem. Technol., **43**, (1970), 1215.
- [31] Chakraborty S. K. and De S. K., Rubb. Chem. Technol., **55**, (1982), 990.
- [32] Kojima Y., Usuki A., Kawasumi M., Okada A., Fukushima Y., Kurauchi T. and Kamigaito O., J Mater Res., **8**, (1993), 1174.
- [33] Lopez - Manchado M.A., Herrero B. and Arroyo M., G.E.Zaikov, A.Jimenez(eds), New developments in polymer analysis, stabilization and degradation, Nova Science Publishers, Inc., New York, (2005), 147.
- [34] ASTM D 471, Standard Test Method for Rubber Property-Effect of Liquids.

- [35] Sir Geoffrey A. and Bevington J.C., Geoffrey C.E., Anthony L., Saverio R. and Pierre S. (ed), *Comprehen. Polym.Sci.* Pergamon Press, New York, (1989), 15.
- [36] Arthur W.E., *Atlas of Polymer Morphology*, Hanser, New York, 1989.
- [37] ISO 10993-5, *Biological evaluation of medical devices-Part 5, Tests for in vitro cytotoxicity*, International Organization for Standardization, Geneva, Switzerland, (1999).
- [38] Fotakis G. and Timbrell J.A., *Toxicol Lett.*, **160**, (2006), 171-177.
- [39] Vega-Avila E. and Pugsley M.K., *Proc. West. Pharmacol. Soc.*, **54**, (2011), 10-14.
- [40] Elizabeth K. A., *Evaluation of natural rubber latex gloves and vulcanizates with special reference to residual zinc dithiocarbamate accelerator*, Ph. D. Thesis, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India, (2005).
- [41] Hyun S. B., Ja Y. Y., Dong K. R., Dong W. H., Dong H. L., Oh-Hun K. and Jong-Chul P. , *Yonsei Med. J.*, 46(4), (2005) , 579-583.

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