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In vitro cytotoxicity study for electron beam cured adhesive tape for bio-medical application

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Abstract

A device made for biomedical applications must be biocompatible and biologically safe for use. The used ingredients may leach off a device into adjacent tissue and can harm the body after or during application. In the present study qualitative and quantitative methods were used to assess the cytotoxicity of electron beam (e-beam) cured polyurethane pressure sensitive adhesive (PU-PSA) tape materials made for different biomedical applications. In the qualitative morphological results, it was found that there was no cell lysis or morphological changes in triplicate culture wells of the extract of e-beam cured PU-PSA tape. In the quantitative method of cytotoxicity studies, MTT cell viability (%) for PSA tape samples were >90, i.e. relative value of toxicity was nil. In case of agar overlay test for test samples, the relative value of toxicity was nil, while in filter diffusion score for the test samples was between 0-0.2, hence the relative degree of toxicity was nil.

Key words

Adhesive tape, Biocompatibility, Cytotoxicity, Electron beam, Polyurethane

Introduction

Submissions for approval of medical devices by regulatory agencies require that biocompatibility assessment be conducted to assure safety of the device or material which can be obtained by testing according to the recommended guidelines of International Organization for Standardization (ISO 10993-1, 2009; ISO 10993-2, 2006; ISO 10993-5, 2009; ISO 10993-12, 2012). Surgical tapes and drapes are being used for a long time for different bio-medical applications. The early PSAs were based on natural or synthetic rubber, polyacrylates, silicones but they suffered from certain limitations *i.e.*, low tack on substrate, excessive water absorption, presence of residual monomer, non biocompatible etc (Singh *et al.,* 2011). The latest trend in PSAs for bio-medical applications suggests that PU based materials may meet the essential requirements and have been reported to offer unique properties not found in other adhesives such as absorption of blood, urine, sweat and saliva, bonding durability being high, adherence to a wide variety of substrates, ability to perform in a wide temperature range, chemical and solvent resistance, variety of options available as per usage (Singh *et al.,* 2012; Singh *et al.,* 2014). It has been reported that PU-based adhesives gain strength by curing at elevated temperature. If the thermal process for making such adhesives is used, it will have limitations such as evaporation of chemical in working area, time consuming process, lack of proper curing, incomplete curing leaving some amount of residues remaining uncured and toxic product (Singh *et al.,* 2013).

In order to ensure that curing is done without any adverse effect on the performance of adhesive instead of using thermal process, environment friendly processing technique e.g., radiation processing can be a better option (Singh *et al.,* 2013; Singh *et al.,* 2014). Radiation processing of PU systems can be carried out by one of the irradiation techniques: UV, e-beam, X-rays and γ-radiation. E-beam cured products have many remarkable advantages (free of toxic residual substances, longer shelf life, weather-resistant, color-stable, resistant to a wide range of chemicals etc) over UV, X-rays and γ-radiation while developing adhesive tape for medical application. A medical grade PSA tape should be good in peel adhesion, shear adhesion, initial tack, moisture vapor

transmission rate, comfortability and biocompatibility (Webster, 1997).

For different biomedical applications, especially delicate parts of human body that come in contact directly with the device used, has become essential that such devices are insured to be biocompatible (Fournier *et al.,* 2003). Among the biocompatibility tests, cytotoxicity is one of the most important study which can be done *in vitro*.

A cytotoxicity test determines whether a product or compound will have any toxic effect due to leachables on living cells. Generally these are used as a screening tool for raw materials or component products before they are put into design of a medical device. In view of the above, the present study aimed to describe the developmental and evaluation of cytotoxic potential of e-beam cured PU-PSA tape by using base resin and other functional ingredients for different medical applications.

Materials and Methods

Genomer 4269, an aliphatic urethane polyester acrylate, in combination with 2-[{(butylamino) carbonyl}oxy]ethyl ester and 2-propenoic acid was procured from Rahn USA Corp., USA. It has a glass transition temperature (T_g) of -15 ºC, viscosity 21 Pa.s and density 1.1g ml^{-1}, water solubility <1g l^{-1} and is used as base resin. Genomer 6043, an inert modified saturated polyester resin with a combination of 2-[{(butylamino) carbonyl}oxy]ethyl ester and 2-propenoic acid having T_g of -18 ^{0}C, flash point >100 ºC, viscosity 19 Pa.s, density 1.13g ml^{-1} and water solubility <1g l^{-1} was procured from Rahn USA Corp., USA and used as tackifier. Isophorone di-isocynate (IPDI), having molar mass 222.3 g mol^{-1}, density 1.06 g cc^{-1}, m.p. -60 ºC, b.p. 158 ºC and flash point 155 ºC was procured from Sigma-Aldrich, USA and used as crosslinker. Fumed silica (Cab-O-Sil) with particle size 5-50 nm, surface area 5-600 m^2 g^{-1} was procured from Cabot, USA and used as thickener. All these materials were used as such without further purification in the adhesive formulations.

L 929 mouse fibroblasts, American Type Culture Collection CCL-1 was for the study and maintained in continuous culture in minimum essential medium (MEM) with 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 5% fetal bovine serum at 37±1 ºC, in air containing 5% CO_2. Cells were passaged with 70-80% confluecy by treating with 0.5g l^{-1} trypsin 0.2g^{-1} l^{-1} ethylene diamine tetraecetic acid (EDTA) in Earl's balanced salt solution (Gibco BRL, UK). Cell viability was determined with trypan blue exclusion test (Berg *et al.,* 1972). Cells were arranged in 96-well culture clusters (Costar, USA), at a density of 15,000 cells/well in 100 µl, incubated for 24 hrs to allow attachment. The medium was changed with 100 µl of test or control medium in air containing 5% CO_2 at 37±1 ºC for 30 min. After 24 hr incubation, cytotoxicity was assessed for the above study.

Preparation and curing of PSA tape : PU-PSA composition was made by combining base resin with other functional ingredients. In order to evaluate PSA for its performance, a PSA tape was made by coating 0.2 mm thick and 30 g m^{-2} adhesive layer on a release paper and left for air drying at room temperature for 30 min. The adhesive layer thus formed was ultimately transferred to the non-woven polypropylene fabric to make PSA tape. Thickness of non-woven fabric was 0.32 mm while its weight was 90 g m^{-2} (Singh *et al.,* 2012).

The dried tape samples were irradiated in air at Bhabha Atomic Research Centre, Mumbai, India, by an e-beam accelerator ILU-6 (Budker Institute of Nuclear Physics, Russia). The accelerator had 2.0 MeV energy level with conveyor speed of 3 cm sec^{-1} for 10 kilogray (kGy)/pass and 6 cm sec^{-1} for 5kGy/pass, with pulse current of 300mA, average current 2mA and pulse frequency of 15Hz. The samples were irradiated at different doses starting from 5 kGy to 60 kGy and 25 kGy was optimized as optimum dose (Singh *et al.,* 2014). The irradiated samples were further kept in vacuum oven at 50 ºC for 1 hr to avoid any kind of entrapment before packaging. Hence, developed e-beam cured PU-PSA tape was then ready for study.

Preparation of extracts : Pieces were cut from PSA tape materials and placed in glass vials having cell culture medium, 6 cm^2 of PSA tape material ml^{-1} medium as per ISO standard (ISO 10993-12, 2012*)*. Agitation was done for 24 hrs in a water bath at 37±1 ºC and filtered using Millex-GS sterile filter, Millipore, France.

Preparation of cell monolayer : The confluent cell monolayer of L-929 cell lines was used for qualitative morphological evaluation of cytotoxicity in the study and cells were rinsed twice with phosphate buffer saline (PBS) and decanted. The culture medium was prepared using serum, antibiotics and aspirated from cell culture flask containing a near confluent cell monolayer. 5.0 ml of trypsin solution was added into the flask and incubated for 2 min at room temperature to suspend the cells. Subsequently, 10 ml media was added for neutralizing the effect of trypsin and cell suspension, centrifuged for 5 min at 700 rpm. The supernatant was discarded and cell pellet was resuspended in 10 ml of fresh media and mixed thoroughly. Finally, a cell suspension of freshly suspended cells was obtained.

One cell suspension was transferred into triplicate culture wells for each test item, positive control (Latex rubber) and negative control United States Pharmacopeia Negative Bioreaction Reference Standard (high density polyethylene). The triplicate culture wells were kept for incubation at 37±1 ºC in an incubator containing 5% of CO_2 until a near 70-80% confluent cell monolayer was formed, as observed by microscopic examination. After 70- 80% confluency was formed in each well of the triplicate culture wells, the growth media from triplicate culture wells was replaced with 2 ml extract of e-beam cured PU-PSA

tape as negative and positive control separately.

All the triplicate culture wells were incubated at 37 ºC in an incubator for 24 hrs in 5% CO_2 atmosphere. After 24 hrs, 2 ml of 0.01 % Neutral Red (Sigma Aldrich, USA) solution was added to each dish (Falcon, USA) and incubated for 1hr. The neutral red solution was poured off and the triplicate culture wells were examined microscopically. The presence of cytotoxic leachates was indicated by loss of cell viability. To test cytotoxicity by direct contact, agar diffusion or overlay assay was conducted by placing the test device or a representative portion directly on mammalian cell layer, protected from mechanical damage by a layer of agar. Cytotoxic leachates diffused into the cell layer via agar and toxicity was indicated by loss of viable cells around the test device. Qualitative morphological grading of cytotoxicity of extracts and confluency of monolayer was recorded as (+) if present and (-) if absent.

MTT test : Quantitative cytotoxicity of PSA tape material was assessed using dimethylthiazol diphenyltetrazolium (MTT) assay (Edmondson *et al.,* 1988; Mosmann, 1983). Optical density was noted at 570 nm, using UV-2600 spectrophotometer (Shimadzu, Japan). Mean test results were calculated and presented as percentage of control cells. Each value represents mean of two experiments, using at least 3 replicates of each extract per experiment. Cytotoxicity was rated based on cell viability relative to controls as severe, moderate and slight or non-cytotoxic (Lönnroth *et al.,* 2001; Lönnroth *et al.,* 2003). The materials were rated, based on their cytotoxicity from highest to lowest.

Agar overlay test : In agar overlay test (Borenfreund *et al.,* 1984), cells were observed under microscope and cytotoxic effect of PSA tape material was noted as lysis of cells subjacent to PSA tape material and decolorization of stained cells. Cytotoxicity level of PSA tape materials was based on the diffusion strength of toxic ingredients in the agar and toxicity of test material to cell membranes.

Filter diffusion test : In the present study (Wennberg, 1988), filters were observed macroscopically and stain intensity of each test specimen contact area was evaluated with the background stain (Berg *et al.,* 1972; Lönnroth, 2005). Each test was carried out twice using at least three replicates for each experiment. Cytotoxicity of PSA tape materials was established, based on a scoring system that takes into account the staining intensity of zone and extension of the affected region. The median values of the observed results were calculated and transformed to a relative degree of cytotoxicity.

Results and Discussion

The present study used qualitative and quantitative methods to assess cytotoxicity of e-beam cured PSA tape materials having pH 7.0, moisture vapor transmission rate (MVTR) 92±1.22 g hr^{-1} m^2, peel adhesion strength 2440 N m^{-2}, shear adhesion strength 13205 N m^{-2}, initial tack 23 N m^{-1} and shrinkage 2.05 %; made for different biomedical applications. The study specifies incubation of cultured cells in contact with extracts of device and determines the biological response of mammalian cells *in vitro* using appropriate biological parameters.

The qualitative morphological results for PU-PSA tape are summarized in Table 1. From the results, it was found that there was no cell lysis or morphological changes in the triplicate culture wells of the extract of e-beam cured PU-PSA tape as compared to positive and negative control. Hence, on the basis of qualitative morphological test results, the extract of test item was found to be non-cytotoxic.

Quantitative relative values of cytotoxicity studies (MTT % cell viability, agar overlay and filter diffusion score) are summarized in Table 3 on the basis of reference Table 2. The MTT test demonstrates the ability of cells to reduce tetrazolium salt to formazan product indicating mitochondrial activity which is seen only in living cells (Berg *et al.,* 1972). The extraction procedure using cell culture media limits the extractants to watersoluble substances. Thus, a strong toxic response in MTT test indicates toxic water-soluble substances released from glove material. The

Table 1 : Summary of results of test item, negative control and positive control

Well	Confluent monolayer	Lysis (%)	Grade	Reactivity
PSA Tape (Test item)				
Test-A	(+)	0	0	None
Test-B	(+)	0	0	None
Test-C	(+)	0	0	None
USP Negative Bioreaction RS (Negative control)				
Negative Control-A	(+)	0	0	None
Negative Control-B	(+)	0	0	None
Negative Control-C	(+)	0	0	None
Latex rubber (Positive control)				
Positive Control-A	(-)	65	3	Moderate
Positive Control-B	(-)	65	3	Moderate
Positive Control-C	(-)	65	3	Moderate

long extraction time (24 hrs) used in this study might not have influenced the response in MTT assay. Studies have shown that the leaching time is important but complete effect of leaching was reached within an hour and longer leaching time did not reduce cytotoxicity (Geertsma *et al.,* 1996). In MTT test, it was found that percent cell viabilities in test and negative control samples were >90, *i.e.,* relative toxicity value in both samples was nil but in case of positive control it was between 30-60 (moderately cytotoxic) (Wennberg, 1988; Cory *et al.,* 1991).

The agar overlay assay demonstrates the ability of a test substance, diffused through the agar layer, to damage plasma or lysosomal membranes of the cells, resulting in a release of the preloaded neutral red dye. Viable cells take up and retain the dye compound (Lönnroth *et al.,* 2001; Lönnroth, 2005). Determination of lysis subjacent to test material and loss of colour of the stained cells, enables relative toxicity to be assessed. In the agar overlay, test material was placed on the agar layer for 24 hrs (indirect contact). In case of agar overlay test for the test and negative control samples, the median values of the results were determined and transformed in to a relative degree of cytotoxicity as shown in Table 3 on the basis of reference Table 2. The lysis index ranges from 0-0.2 *i.e.* the relative toxicity value in both the sample was nil, but in positive control sample it was found between 2.2-2.5 which come in the category of moderately cytotoxic (Ikarashi *et al.,* 1992).

Table 2 : Cytotoxicity tests expressed in relative values

	Relative degree of cytotoxicity			
Test method	**None**	**Slight**	**Moderate**	**Strong**
MTT (cell viability, %)	>90	60-90	30-60	<30
Agar overlay (lysis index)	0-0.5	0.6-1.9	2.0-3.9	<4.0-5.0
Filter diffusion (score)	0-0.4	0.5-1.4	1.5-2.4	2.5-3.0

Table 3 : Summary of results

	Name of the tests		
Particular of samples	**MTT (Cell viability, %)**	**Agar overlay (Lysis index)**	**Filter diffusion (Score)**
PSA Tape (Test item)			
Test-A	>90	0.2	0.2
Test-B	>90	0.1	0.0
Test-C	>90	0.2	0.1
USP Negative Bioreaction RS (Negative control)			
Negative Control-A	>90	0.0	0.1
Negative Control-B	>90	0.2	0.1
Negative Control-C	>90	0.2	0.2
Latex rubber (Positive control)			
Positive Control-A	30-60	2.5	1.8
Positive Control-B	30-60	2.4	1.9
Positive Control-C	30-60	2.2	1.8

The filter diffusion test demonstrates the ability of cells to transform a yellow succinate solution to a blue furate product indicating mitochondrial activity, which is seen only in living cells. Determination of succinate dehydrogenase in cells exposed to test substances as compared to control enables relative toxicity (Wennberg, 1988). In filter diffusion test, cells were in contact with the glove materials and they were separated only by a filter. The close contact between cells and test material enhanced the possibility that all leachables and not only water soluble substances, would reach the cells (Lönnroth 2003). The results for filter diffusion test are also summarized in Table 3 on the basis of reference Table 2. From the results of the study, the filter diffusion score for test and negative control samples were between 0-0.2, hence the relative degree of toxicity was none but for positive control ranged between 1.8-1.9, which was moderately toxic in nature. From the results, it is clear that all the positive control materials released substances with the ability to penetrate through a filter and induce toxic effect on cell functions, while the remaining materials (test and negative control) were non-cytotoxic in nature (Wennberg, 1988).

In cytotoxicological methodology MTT cell viability (%) for PSA tape samples were >90, *i.e.* relative value of toxicity was nil. In case of agar overlay test for test samples, relative toxicity value was nil, while in filter diffusion score for test samples was between 0-0.2, hence the relative degree of toxicity was nil. It might be due to the fact that materials released substances with the ability to diffuse through a filter and exhibited toxic effect on cell functions as shown in the study. The remaining materials showed non- or slight cytotoxicity.

The present study used three different methods to assess cytotoxicity of e-beam cured PU-PSA tape materials made for different medical applications. The mode of exposure and exposure time differed. In MTT assay, cells were exposed to extracts of materials for 4 hrs. In filter test, cells were exposed for 2 hrs to substances released from the materials whereas in agar overlay test, substances released from the material for 24 hrs diffuse through agar layer before reaching the cells. On comparing the results of these three tests a similar degree of toxicity for all positive control, negative control and PU-PSA tape materials was observed.

From the triplicate test results of e-beam cured PU-PSA tape as compared to positive and negative control, the extract of test item was found to be non-cytotoxic. Hence, based on qualitative and quantitative test results, the given sample of e-beam cured PU-PSA tape meets the requirement of test, conducted as per specification.

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