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Minimising Chemical Hazards to Improve Biocompatibility of Natural Rubber Latex Products

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This study examines the residual rubber chemicals commonly used in natural rubber (NR) latex formulation and their effects on the biocompatibility of finished products. Several in-house compounds differing in chemical compositions were prepared and analysed for their physical properties, residual chemicals and biocompatibility. From this, an optimum formulation with low residual chemical content and acceptable physical properties was selected as a model design. In vitro cytotoxicity elution tests with L 929 fibroblasts showed all sample extracts to be poorly biocompatible. This however improved markedly when tested against extracts made from solid samples that had undergone a pre-wash in acetone. Tensile strength and M300 values of the solid samples were within an acceptable range with minimal changes after the acetone wash. Residual chemical content of the extracts was markedly reduced when analysed using high performance liquid chromatography based on the diethyldithiocarbamate (DEC) derivative method. A further study on 12 commercial powder-free NR examination gloves showed a similar improvement after the acetone wash where cell viability of L 292 fibroblasts was substantially increased in tandem with reduced residual chemical content. A genotoxicity study on Chang liver cells via the Alkaline Comet Assay showed minimal DNA damage. The physical properties of these gloves remained unaffected after the acetone wash.

Keywords: natural rubber; residual chemicals; biocompatibility; physical properties

Public concern over health issues related to natural rubber (NR) products began to escalate in the early 1980s, particularly with regards to the Type I allergy caused by the innate NR proteins[1–3]. Type IV allergy became a focal point later on, attributed predominantly to rubber chemical additives[4]. Among these, the accelerator zinc diethyl dithiocarbamate was identified as the most allergenic inducer compared to other rubber chemicals[5]. While toxicity of rubber chemicals are well documented[6–10], a point noteworthy is that some of these studies were done directly on the chemicals, whereas in reality, these chemicals are added together with other materials where the remote effects of individual chemicals have likely combined into a different synergistic effect.

Hence, it is equally important firstly to study the total residual chemicals released from the finished products, followed later

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by identification of the individual chemicals where possible. Residual chemicals are very much influenced by the effectiveness of their integration into the system of the compound in making, while exhaustive uptake and a stable compound will understandably give rise to low chemical residues. This coupled with washing of the finished products, is the desired attributes of medical device manufacture.

Notwithstanding this, leaching of residual chemicals from medical devices has been reported to incur medical complications such as cytotoxicity and tissue damage[11] as well as other adverse biocompatibility issues[12–14]. Other examples include the release of chemical additives from rubber syringe closures, gloves and catheters[15–17]. Dithiocarbamate accelerators have been found to impede cell growth and proliferation, the extent of which correlated inversely with the chain length of the alkyl moiety[6]. While most of these studies were on NR products, toxicity associated with synthetic materials such as polyurethane and silicone was not entirely absent[10,18]. This clearly projects the superior role of residual chemical additives on the toxicity of products compared to the raw material itself.

To date, extraction procedures to quantify these residual chemicals remain limited[19–21]. Some later developments involving acetone extraction, evaporation and reconstitution[22] have been widely used as these methods gave the best yield even though they did not depict the actual *in-use* condition. Despite the setback in measurement methods, minimising residual chemicals in NR medical devices is arguably still the ultimate goal towards achieving biocompatible medical devices. While rubber chemicals are used in NR product manufacture to impart superior properties, residues of these chemicals are invariably found in the finished products, some of which have been reported to trigger allergic response in sensitised users[16,23,24].

To address concerns over the safe use of NR products particularly NR medical devices, a study on minimising the chemical residues was thus carried out. The aim is to relate the effect of residual chemicals on the biocompatibility and functionality of NR materials, with the hope of developing biocompatible formulations for high-end NR medical applications.

MATERIALS AND METHODS

Materials

Commercial concentrated high ammoniated latex (HA) and industry grade chemicals were used throughout the study. Twelve commercial powder-free NR latex gloves from different manufacturers were also evaluated.

Sample Preparation

For the preliminary study, *in-house* dipped films were prepared with minimal content of chemicals to study its cytotoxic effects (*Table 1*). Eight compounding formulations were prepared as tabulated in *Table 2*. The chemicals were added in the sequence as shown. Compounded latex was stirred for 60 minutes and left to mature at room temperature for 48 hours. Dipped NR latex films coagulated using calcium nitrate, were prepared from the matured NR latex mix. The NR films were leached during their wet-gel state in a hot water bath at 70 ± 2ºC for 1 minute. The films were further heated in a circulating air oven at 100ºC ± 2ºC for 30 mins, before being leached in a hot water bath for 1 min, and finally dipped into a 10% corn starch slurry solution. The dipped film was then air-dried and stripped from the glass former. Test specimens were then prepared from these natural rubber latex (NRL) films.

TABLE 1. FORMULATION FOR NR LATEX DIPPED FILM

Component	Role	Ingredient	p.p.h.r.*
Basic compound	Raw material	60% HA	100
	Stabiliser	10% KOH	0.3
	Stabiliser	10% Laurate	0.3
	Antioxidant	40% Wingstay L®	1.5
Curing agent	Vulcanisation	50% Sulphur	0.10
		50% ZDEC	0.05
		50% ZnO	0.03

* p.p.h.r. denotes parts per hundred of rubber

TABLE 2. COMPOUNDING VARIATION FOR NR LATEX DIPPED FILMS

Compounding	Basic compound	50% Sulphur	50% ZDEC	50% ZnO
1	√	-	-	-
2	√	√	√	√
3	√	√	-	-
4	√	-	√	-
5	√	-	-	√
6	√	√	√	-
7	√	√	-	√
8	√	-	√	√

Following findings from the preliminary study described earlier, the subsequent stage involved preparing *in-house* dipped films using a formulation which has been identified to give optimum physical properties for NR latex application with minimal chemical residues[25]. The formulation used is given in *Table 3*. Dipped films were prepared according to previous experimental practices.

In addition to these *in-house* dipped films, 12 different brands of commercial NR examination gloves were selected for this study as coded in *Table 4*. Both the *in-house* dipped films and gloves were evaluated for their physical properties, residual chemicals, cytotoxicity and genotoxicity.

Acetone Extraction and Physical Testing

Before the subsequent tests were carried out, all samples were first treated in two different conditions namely, with and without acetone wash. This procedure was carried out according to the *ASTM D297-93(2006)*[26]. For the tensile properties, *ISO 37*[27] practices were followed where the tensile strength and modulus at 300% elongation (M300) were measured.

Chemical Residues Extraction

This method was carried out to extract the chemical residues, specifically the DEC-type

TABLE 3. FORMULATION FOR NR LATEX VULCANISATION MIXES

Ingredients	p.p.h.r.
60% HA latex	100
10% Potassium hydroxide	0.3
20% Potassium laurate	0.3
50% Sulphur	1.0
50% ZDEC	0.9
50% ZnO	0.4
40% Wingstay L®	1.5

(dithiocarbamates) from the NR samples. Azeotrope mixture (AM) consisting of 582 mL chloroform, 704 mL acetone and 548 mL methanol was used. A test specimen of 7 cm × 7 cm was first immersed in a vial containing AM at a ratio of 1:10 (w/v). The vial was then placed on a horizontal shaker and continuously shaken at a rate of 200 r.p.m. for 3 h under ambient conditions. The test specimen was removed and the AM in the vial was dried overnight in a fume cupboard followed by final drying in a vacuum oven at 55 ± 2ºC, to collect the dried residues.

High Performance Liquid Chromatography (HPLC) Analysis

The dried residues from the extraction process were redissolved in 5.0 mL chloroform, mixed with 1.7 mL copper sulphate solution (1M) and shaken to form the copper diethyldithiocarbamate (CuDEC) complex[28]. The complex formed was quantified against standard solutions by HPLC method. The instrument used was a Waters HPLC equipped with C18-bonded reversed phase column (3.9 micron, 150 mm) at a UV-wavelength set at 269 nm. The gradient mobile phase used was acetonitrile and water at a ratio of 90:10 (v/v) with a 1.0 mL/min flow rate.

Surface Analysis

Dipped *in-house* NR films with and without acetone wash, were examined using the Carl Zeiss Image Analyzer at 20X magnification, to visualise the effect of acetone on the film surfaces. Atomic Force Microscopy (AFM) was carried out at 0.75 Hz scan rate with 512 scan points to obtain the surface topography of the film surface.

TABLE 4. COMMERCIAL EXAMINATION GLOVES

Sample No.	Sample Code
1	1
2	3
3	7
4	14
5	19
6	21
7	23
8	31
9	33
10	35
11	38
12	41

Biocompatibility Tests

Samples were tested for their effects on *in vitro* cultured cells to examine the extent of cell toxicity and DNA damage. For cell toxicity, samples were extracted in culture media, and the cytotoxicity elution test was carried out as described before[29] by exposing *L 929* murine fibroblasts (obtained from the *American Type Culture Collection*) to the extracts for 24 h at 37ºC and 5% CO_2 in an incubator. This was followed by reaction of the exposed cells to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or more commonly known as the MTT assay[30]. Measurement of cell viability is based on the conversion of tetrazolium salt to formazan by the dehydrogenase enzymes found in living cells. Formazan crystals were solubilised overnight and the absorbance reading was measured at 570 nm.

For the study on DNA damage, the genotoxicity test *via* the Alkaline Comet Assay was used. Briefly, Chang normal liver cells were exposed to the sample extracts, followed by mixing the reacted cells with agarose and setting the mixture onto glass slides. The slides were then placed in lysing solution to promote unfolding of DNA. The slides were next transferred into a horizontal gel electrophoresis bath and left to stand in buffer for 20 mins to allow unwinding of the DNA. Electrophoresis was carried out for 20 mins at 25V, after which the slides were washed in Tris buffer at pH 7.5, followed by staining with etidium bromide. Observation was made using fluorescent microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Photomicrographs were taken at 400X magnification. DNA migration was determined using the '*TritekCometScore*' software by measuring the nuclear DNA and the migrating DNA on 50 randomly selected cells in each exposure.

RESULTS AND DISCUSSION

Optimisation of NRL Processing

In the earlier stage of this study, dipped films were produced with minimal amounts of chemical ingredients to identify the optimum formulation. Eight compounds were prepared by varying the curing agents (*Table 2*) to study the effect of minimal chemical content on the toxicity of cells. Curing agents were selected for study as these were known to play a more deciding role in toxicity compared to rubber chemicals used for other functions[6,7,29].

Results in *Table 5* show that all films including the control, failed the cytotoxicity test with Grade 4 under the *United States Pharmacopoeia (USP)* Grade System (elution test). A Grade 4 denotes severe toxicity where a nearly complete cell death is observed. Generally, a sample is considered having some extent of toxicity when the monolayer cell formation is poor, and the cells are rounded with punctuated membrane and poorly defined intracellular content. In some occasions, enlarged cells may also be seen. In contrast, a biocompatible material encourages good cell growth and proliferation, and the *L 929* cell monolayer is uniform with well defined spindle shaped cells.

While the control sample contained only basic compounds without the curing agents, observation from this study indicates that the basic compounds also have the potential to trigger cell toxicity. One reason for this could be the antioxidant used, which is reported elsewhere to display relatively weaker toxicity than DEC-type accelerator[6,7,29]. Interestingly, in an earlier study with HA film extract where no chemical except ammonia preservative was used, more than 80% of surviving cells were observed (*Figure 1*)[31]. Similar low toxicity was observed with irradiated (IR) and peroxide-cured (PX) where no rubber chemicals were

TABLE 5. CYTOTOXICITY RESULTS OF IN-HOUSE DIPPED NR FILMS WITH AND WITHOUT ACETONE WASH

Compounding	Without Acetone Wash*		With Acetone Wash*	
	Grading	Pass / Fail	Grading	Pass / Fail
1	4	Fail	2	Pass
2	4	Fail	2	Pass
3	4	Fail	2	Pass
4	4	Fail	2	Pass
5	4	Fail	2	Pass
6	4	Fail	2	Pass
7	4	Fail	2	Pass
8	4	Fail	2	Pass

*Cytotoxicity test was done on neat extracts of the samples.

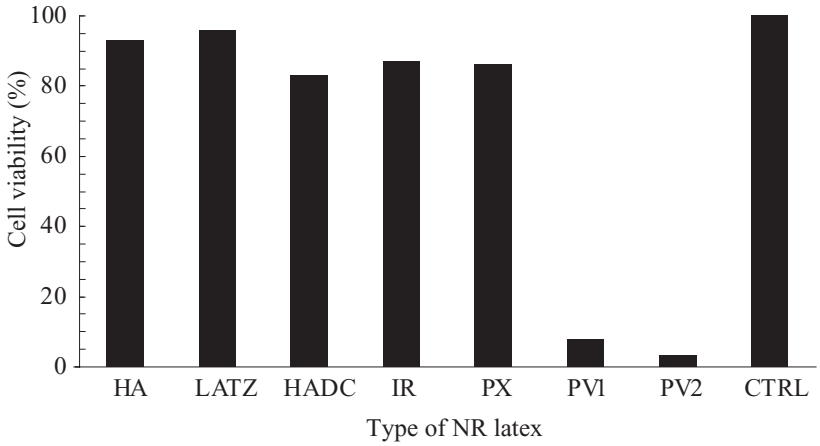


Figure 1. Cytotoxicity of natural rubber (NR) latex from various latex processing systems. Labels denote the following: HA, high ammoniated; LATZ, low ammoniated; HADC, double-centrifuged HA; IR, irradiated; PX, peroxide-cured; PV1 & PV2, sulfur-cured; CTRL: negative control (culture media only).

used. In contrast, adverse toxicity was seen in sulfur-cured latex (PV1 and PV2) where compounding chemicals have been added. This clearly shows that the compounding chemicals rather than the NR latex are the dominant cause of toxicity in *in vitro* cell culture study. However, whether these chemicals exert their toxicity in a remote or synergistic effect is to be identified.

Following the findings described earlier, many attempts were made on the latex formulation to minimise the cytotoxic effect, nevertheless no solution was successful. In view of this, the study took another direction, looking into the processing stage where it was found that washing the compounded and cured films with acetone gave some affirmative findings. Washing with water was earlier

carried out, but this has failed to remove the cytotoxic effects on culture cells. However, it was observed that films washed with acetone for 1 hour at room temperature improved the survival rate of the cells. All samples passed the cytotoxicity test at Grade 2 (*Table 5*). This grade signifies mild reactivity where more than 50% of cells are found to be living. A sample meets the requirement of the cytotoxicity test if it is found to give no greater than a mild reactivity (*i.e*. at minimum Grade 2). This observation clearly shows that the acetone wash was able to improve biocompatibility of the samples. Cell death was evidently caused by residual chemicals in the samples, which were later removed during the acetone wash.

Development of Biocompatible Latex Formulation

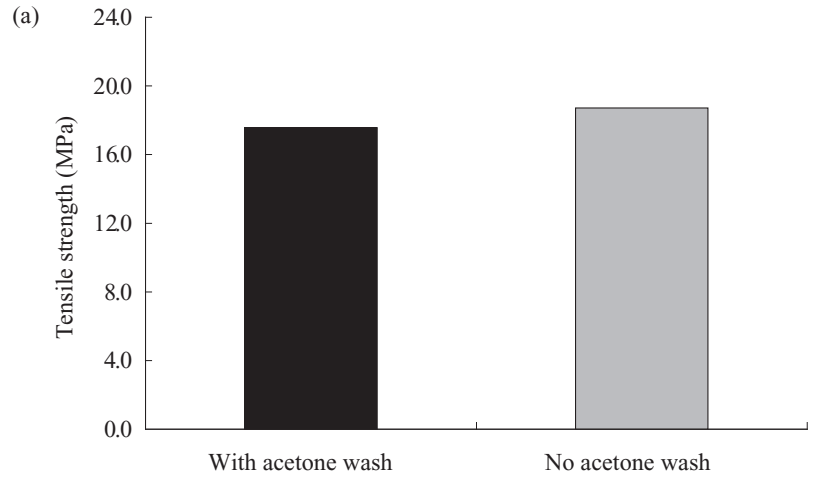
Based on findings in this study, a provisional latex formulation was next designed using optimum amount of chemical ingredients. This formulation was expected to give acceptable physical properties and low amount of residual DEC. *Figure 2a* and *Figure 2b* show that with this latex formulation, there were minimal differences between samples washed with and without acetone for both the tensile strength and M300 values respectively. This indicates that acetone has no measurable adverse effect on the physical properties of the samples under the current experimental conditions.

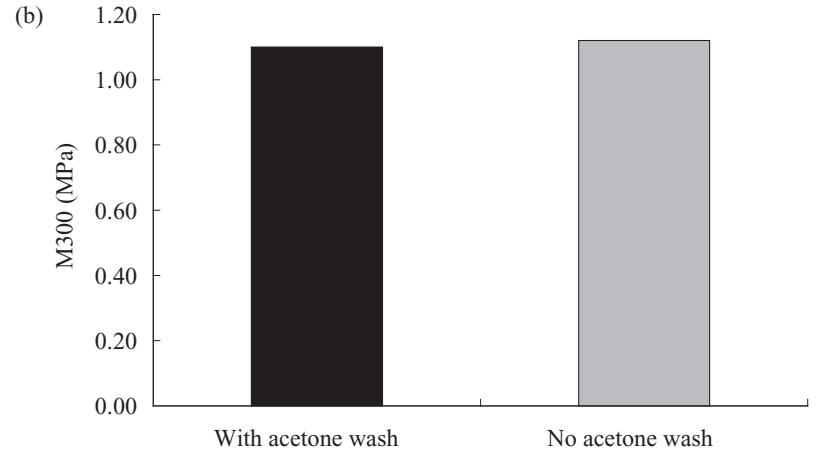
Considering the case of residual chemical DEC, a film that has been washed with acetone eluted almost 60-fold less residual DEC compared to the non-acetone washed film (*Figure 2c*). This suggests that during the wash stage, acetone was able to dissolve and remove the chemical residues and subsequently removed them from the samples surface. An earlier study using distilled water wash failed to elicit a similar effect, probably because the chemical residues were poorly soluble in water.

When the samples were examined under light microscopy, *Figure 3* shows that the film treated with acetone has a smoother surface compared to the non-acetone washed film. The non-treated film was evidently precipitated with a myriad of substances on its surface, and expectedly most would be residual chemicals aside from other nominal artifacts. The acetone washed film was remarkably cleaner; attributed to the removal of residues trapped on the film surface during the washing process, resulting in an even film surface. This observation confirms the superior ability of acetone in removing residual substances from the NR surface. Expectedly, the non-acetone washed film showed a rougher surface due to trapped residues.

Surface topography analysis using AFM indicates that acetone has likely penetrated into the film surface and extracted the residues from the samples. This was seen in *Figure 4a* which shows a relatively even surface of the unwashed film, contrasting the markedly uneven surface topography seen in acetone washed sample (*Figure 4b*). The resulting rougher surface after acetone wash could be one reason for the slight reduction in physical properties as seen in *Figure 2a* and *Figure 2b* mentioned earlier.

As expected, cytotoxicity test shows that the non-acetone washed films elicited severe cell death (Grade 4) whereas with the acetone washed films, up to Grade 1 USP biocompatibility index was observed (*Table 6*). Grade 1 indicates more than 80% viable cells and is taken as having passed the cytotoxicity test. For this part of the study, toxicity was tested at two different concentrations (*i.e.* neat and 10X dilution) in case no distinct result was observed with one concentration. Comparing cell performance at these two concentrations





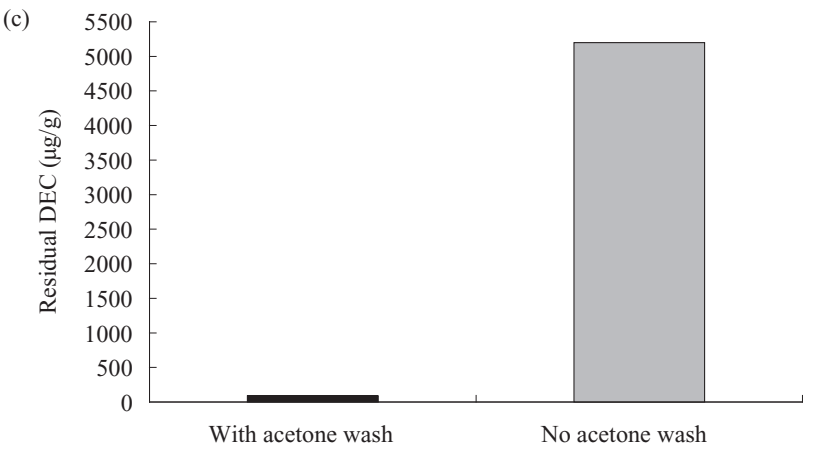
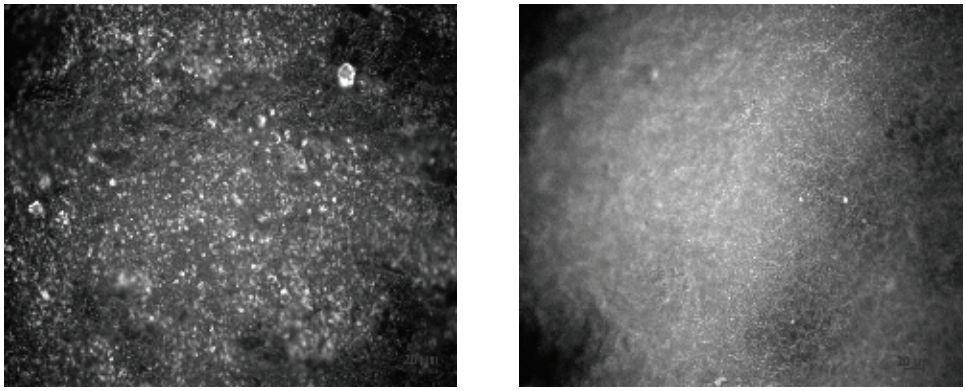


Figure 2. Effect of acetone wash on the (a) tensile strength, (b) M300 and (c) residual DEC content of NR dipped films.



(a) (b)

Figure 3. Micrographs showing differences in dipped film surfaces (a) without acetone wash and (b) after acetone wash. Images were taken using Carl Zeiss Image Analyser at 20X magnification.

TABLE 6. CYTOTOXICTY RESULTS OF NR DIPPED FILMS MADE FROM A PROVISIONAL LATEX FORMULATION WITH OPTIMUM CHEMICAL CONTENT

Sample	Neat Extract		10X Dilution	
	Grading	Pass / Fail	Grading	Pass / Fail
Acetone washed	4	Fail	1	Pass
No-acetone washed	4	Fail	4	Fail

is adequate to gauge improvement in biocompatibility. Cell compatibility was found to be poor for the neat extracts including the sample with acetone wash; the exact reason for this has yet to be identified. However, at 10X dilution, the sample pre-washed in acetone showed marked improvement (Grade 1) while the sample without acetone wash still elicited severe toxicity (Grade 4). This indicates that to some extent, acetone wash has alleviated cell toxicity.

An interesting observation here is results with the 8 in-house compounds described earlier showed a pass in cytotoxicity test (*i.e*. Grade 2) for all the acetone washed samples when tested as neat extracts (*Table 5*). However, with this provisional formulation, the neat extract failed to elicit same effect and passed only when tested at 10X dilution. This is likely because of a greater amount of curing agents used in the provisional formulation, possibly leading to more chemical residues adverse to cells.

Evaluation of Commercial Gloves

An evaluation on commercial gloves was carried out next to examine whether there were differences compared to the *in-house* samples. Twelve powder-free commercial NR examination gloves were pre-washed with acetone and measured for their physical properties, chemical residues and biocompatibility. *Figure 5* and *Figure 6* show

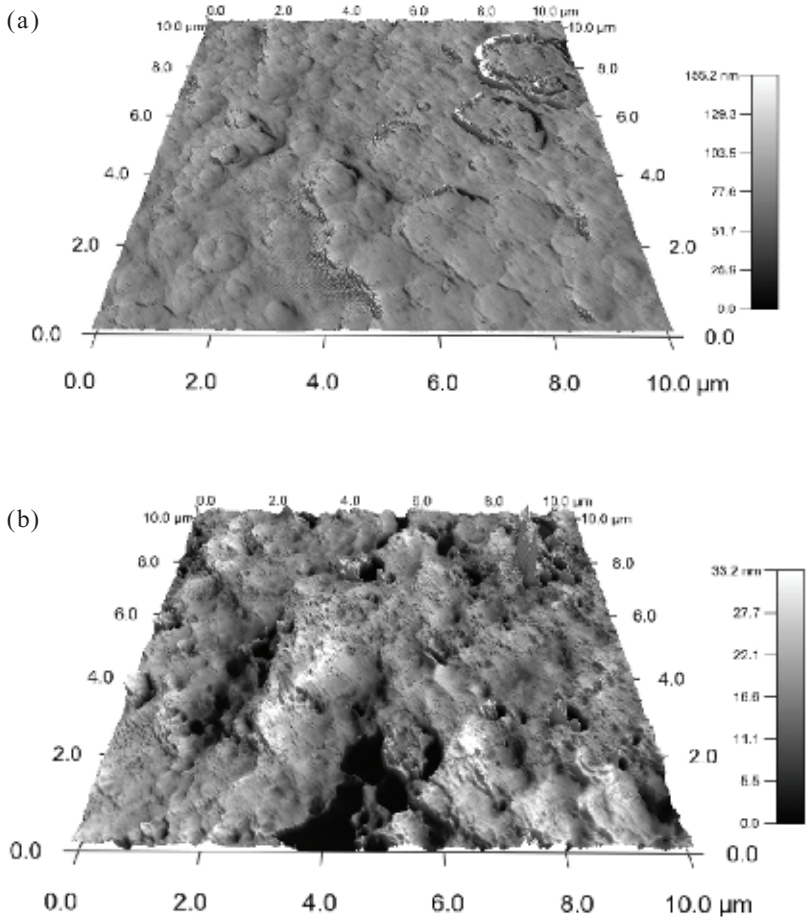


Figure 4. Surface topography of NR dipped film surfaces (a) without acetone wash and (b) after acetone wash. Images were taken using Atomic Force Microscopy at 0.75Hz scan rate.

that the tensile strength and M300 values respectively were not affected much by the acetone wash, as similarly observed with the *in-house* dipped film samples. This indicates that acetone wash carried out on these commercial samples did not exert appreciable adverse effects on their physical performances under the present experimental conditions.

As for the residual DEC, gloves that were washed with acetone prior to test, gave markedly lower residual DEC content, minimally over 90% reduction (*Figure 7*). Interestingly, minimal or no DEC was detected in samples 1, 14, 23 and 33, even without the acetone wash. It is believed that for these samples, either extremely low or no DEC-type accelerator was used in their gloves compounding formulations. It is also possible that these manufacturers practiced more efficient washing steps whereby the surface residual chemicals could have been removed.

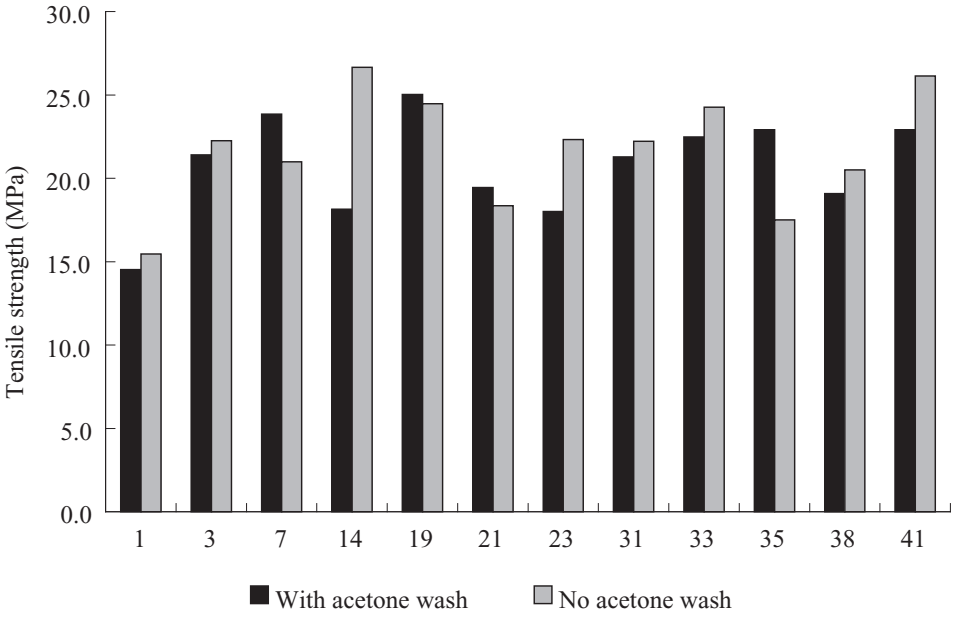


Figure 5. Tensile strength values of commercial gloves with and without acetone wash.

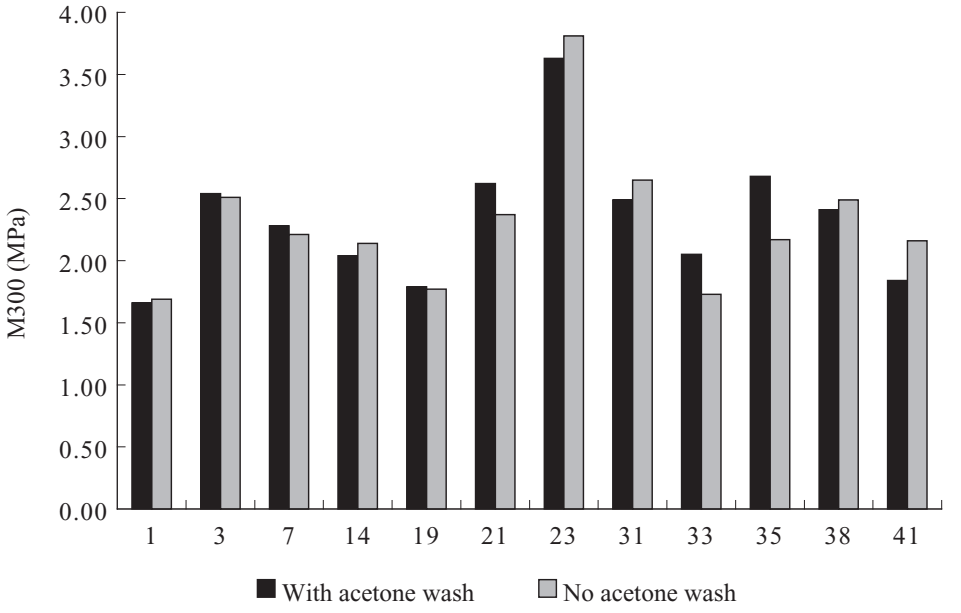


Figure 6. M300 values of commercial gloves with and without acetone wash.

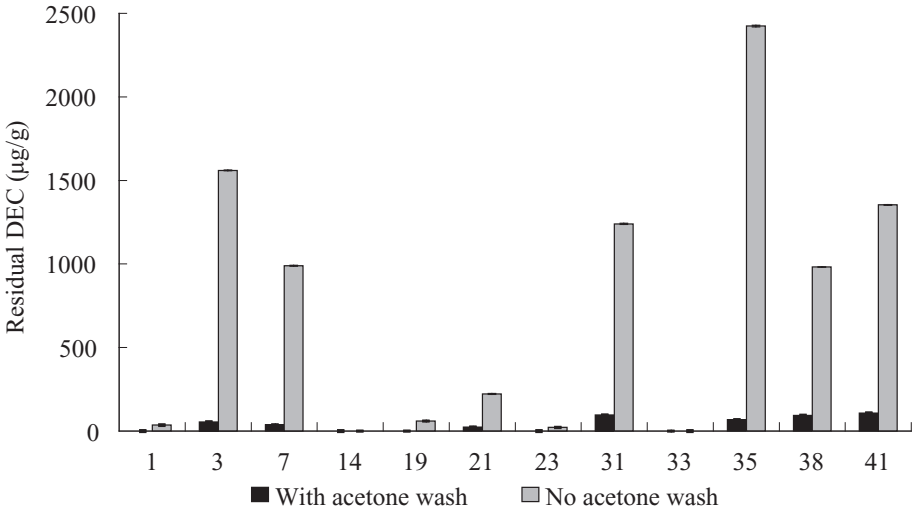


Figure 7. Amount of residual DEC from gloves with and without acetone wash analysed using the High Performance Liquid Chromatography method.

However, the exact formulations could not be obtained from the manufacturers due to propriety rationales. It should be noted that the HPLC method used in this study detects only derivatives from the DEC group. In general, while DEC reduction was obvious after the acetone wash, the extent of this reduction was erratic, likely because of the different formulations and processes used by different glove manufacturers.

As expected, *Table 7* shows that almost all samples failed to pass the cytotoxicity test when no acetone wash was carried out. This improved markedly after the commercial gloves were washed with acetone, where a minimal Grade 2 biocompatibility index was recorded (*Table 8*). This is in agreement with the large reduction in residual chemical content as described earlier. Again, an interesting observation here is samples 14, 23 and 33, where their non-acetone washed samples were the only ones that passed the toxicity test at 10X dilution (*Table 7*). Coincidentally they were also the same samples which showed extremely low residual chemicals prior to acetone wash. This clearly shows that chemical residues play a critical role in material toxicity. A noteworthy observation is sample 1, which was found to be toxic to cells although it has low chemical residue content before the acetone wash. It is possible that this toxicity was caused by other types of chemical not under the DEC group, which could not be detected using the method described in this study.

Genotoxicity study on the same samples using the Comet assay is illustrated in *Figure 8*. The extent of DNA damage is measured based on the relative % DNA between the head and tail following a single cell gel electrophoresis of the exposed cell. Tail intensity below 5 is considered non-genotoxic. Results show that except for samples 23 and 33, the remaining 10 commercial glove samples showed markedly reduced genotoxicity following the acetone wash. This again demonstrates the importance

TABLE 7. CYTOTOXICITY OF COMMERCIAL GLOVES WITHOUT ACETONE WASH. (HIGHLIGHTED SAMPLES INDICATED PASSES)

Sample Code	Neat Extract Grading	10X Dilution Pass / Fail	Grading	Pass / Fail
1	4	Fail	4	Fail
3	4	Fail	4	Fail
7	4	Fail	4	Fail
14	4	Fail	2	Pass
19	4	Fail	4	Fail
21	4	Fail	4	Fail
23	4	Fail	1	Pass
31	4	Fail	4	Fail
33	4	Fail	1	Pass
35	4	Fail	4	Fail
38	4	Fail	4	Fail
41	4	Fail	4	Fail

TABLE 8. CYTOTOXICITY OF COMMERCIAL GLOVES WITH ACETONE WASH

Sample Code	Neat Extract		10X Dilution	
	Grading	Pass / Fail	Grading	Pass / Fail
1	4	Fail	2	Pass
3	4	Fail	2	Pass
7	4	Fail	2	Pass
14	4	Fail	2	Pass
19	4	Fail	2	Pass
21	4	Fail	2	Pass
23	4	Fail	2	Pass
31	4	Fail	2	Pass
33	4	Fail	2	Pass
35	4	Fail	2	Pass
38	4	Fail	2	Pass
41	4	Fail	2	Pass

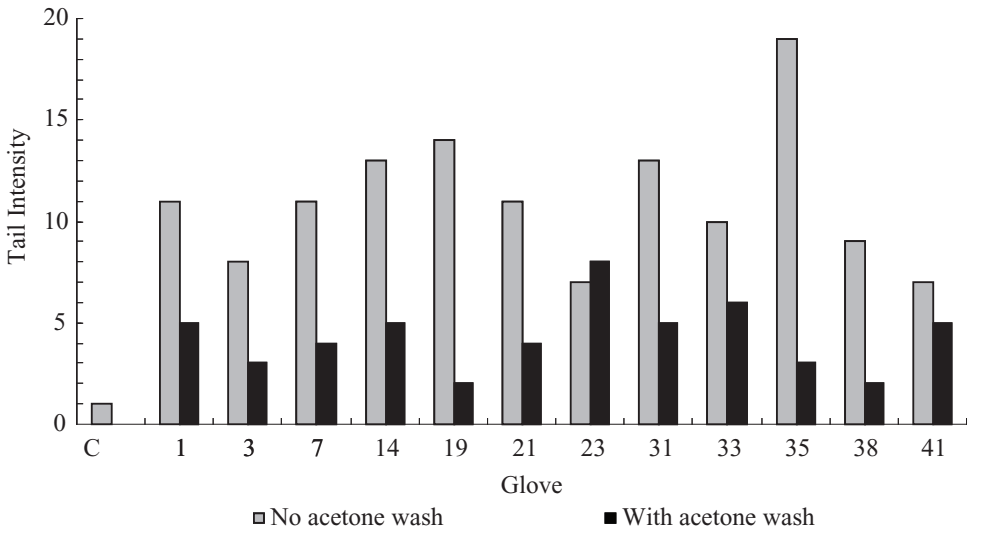


Figure 8. Genotoxicity of commercial glove samples with and without acetone wash analysed using the Alkaline Comet assay. A tail intensity of less than 5 is accepted as negligible DNA damage.

of removing residual chemicals for improved biocompatibility. Ironically where samples 23 and 33 are concerned, these were also two of the only three samples which passed the cytotoxicity test described earlier. While the exact reason for this is yet to be identified, it is possible that these 2 samples might contain chemicals in type or quantity that were specifically damaging to DNA but less detrimental to cell growth and proliferation measured in the cytotoxicity test.

SUMMARY

Our biocompatibility studies in the past have consistently shown that it is very difficult for the sulfur based vulcanised materials using the current generic curing agents, to pass the cytotoxicity elution test even at diluted concentrations. It is quite clear that the road ahead is difficult and long in the quest to develop a biocompatible latex formulation based on the sulfur-vulcanisation process. This is due to the myriad of rubber chemicals involved, some of which are evidently not well tolerated by living cells. Findings from this preliminary study clearly show that residual chemicals are the dominant cause of poor biocompatibility, and post-product processes such as acetone wash or likely other solvents, could effectively remove these chemicals from the NR medical devices, rendering them more biocompatible. This is indeed a critical indication of tangible and improved biocompatibility. While no apparent compromise in physical properties of the samples was seen, its long-term effect is yet to be ascertained. Likewise, environmental implication of such measure also needs to be considered. Use of biocompatible chemicals, efficient chemical integrations or effective washing of end products is likely the viable direction towards developing a biocompatible formulation for high-end medical device application.

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