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Structural and Compositional Characterization of Silverfil Amalgam

Chanthiriga Ramasindarum, Vengadaesvaran Balakrishnan,
Noor Hayaty Abu Kasim and Mohd Ambar Yarmo

Abstract Silverfil™ (Silverfil dental Products, Malaysia) is a commercially available dental amalgam which is based on silver (Ag) and mercury (Hg) only. The objective of this study was to determine the structural state and chemical composition of the starting materials and presence of mercury in the resulting amalgam. All the starting materials of the Silverfil amalgam were characterized using Field Emission Scanning Electron Microscopy (FESEM-EDX), X-Ray Diffraction (XRD) and X-Ray Photoelectron Spectroscopy (XPS). Analysis using FESEM depicts that amalgam (Ag₂Hg₃) clusters were formed as a matrix consisting of crystalline solid. The EDX analysis showed that all the chemical composition of the starting materials consists of Ag and Hg only. The XRD analysis also revealed that Ag and Hg form as isometric (cubic)-hexoctahedral with unit cells of 10.06 Å. This value is similar to a naturally occurring mineral called “Moschellandsbergite” which is found in the district of Landsberg in Germany. The XPS analysis showed that there was no free mercury (Hg⁰) present in the starting material. When the resulting amalgam was analysed, the binding energy of

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Hg (4f) photoelectron peaks were at 101 eV and 100.3 eV confirming the presence of bonded mercury with silver alloy (Ag_2Hg_3). These studies show that there is no free mercury present in Silverfil amalgam.

Keywords Amalgam · Surface analysis · XRD · XPS · Mercury · Silver

1 Introduction

Dental amalgam is the most commonly used dental filling material for over 150 years now due to its low cost, ease of application and the fact that it conforms to the shape of the cavity upon hardening. It is resistant to fracture and durable at room temperature [1, 2]. However, the usage of mercury in dental amalgam is a controversial issue since the 19th century. On 10 June 2010, the U.S. Food and Drug Administration announced plans to hold an advisory panel on 14–15 Dec 2010, to discuss several scientific issues that may affect the regulation of dental amalgam, used for direct filling of carious lesions or structural defects in teeth. The panel has yet to decide as it is hard to separate the risk assessment from the clinical studies. Most of the members believed current studies and reanalysis of earlier studies should inform FDA's risk assessment. Further, the panel discussed alternative composite options to use in patients but pointed out that these alternative options may carry their own risks and data on such alternatives is limited. The panel also suggested labeling changes including the addition of warnings for young children, pregnant women and metal allergies and clearer reference to limitation in the current data. Recently, press release Newswire has reported that an amalgam groups today applauded a move by the United States Government to support the phase down of dental mercury tooth filling by educating the patients and parents about amalgam in order to protect children and fetuses and also to train dental professionals on environmental impacts of mercury in dental amalgams [3]. Studies have shown that mercury is released from dental amalgam and absorbed into human body through inhalation, ingestion and concerns have been raised about the possible toxicity [4–12]. In order to overcome these issues, more studies need to be done with respect to the surface of the amalgam using X-ray photoelectron spectroscopy (XPS), Auger electron spectroscopy (AES) and X-Ray diffraction (XRD). These studies can reveal the relationship between mercury and amalgam [13–16]. In 1987, Hanawa et al. [13] concluded that the concentration of mercury in amalgam after aging was depleted due to the diffusion of composition of zinc and tin with oxygen performing a thin layer on the surface of the amalgam. In 1997, Nakajima et al. [14] revealed that the formation of indium and tin oxide film react as a barrier to reduce the mercury release. Another study achieved by Motohiro et al. [15] on surface analysis of dental amalgam showed that increase in zinc or indium content in zinc or indium containing amalgam forms an oxide layer at the surface to suppress the release of mercury. In 2005, Talik et al. [16] carried out a study on dependencies of seven high copper dental amalgams and mercury

release behavior. They concluded that the amount of Ag, Cu, Sn determines the main properties of high copper amalgams and plays an important role in mercury evaporation. All the studies showed that elements such as indium, zinc, copper and tin, form an oxide layer to restrain the mercury release.

However, Silverfil amalgam (Silverfildental Products Sdn Bhd Malaysia) claim is that this product consists of only silver and mercury, which form gamma-1 (γ_1) crystal without producing any free mercury. This product has been accepted by the Ministry of Health Malaysia as the replacement restorative material for traditional amalgam. The aim of this study is to prove that the silver element does bind together with mercury after trituration without the release of any free mercury. The structural behavior of this amalgam was also investigated using scanning electron microscope and elemental analysis.

2 Materials and Methods

2.1 Preparation of Amalgam

The starting materials and amalgam used were obtained from Silverfildental Products Sdn Bhd. Kuala Lumpur, Malaysia: treated silver (Ag), silver mercury (Ag_3Hg_2), mercury (Hg) and Silverfil amalgam polymer capsules. The treated silver (Ag), silver mercury (Ag_3Hg_2) and dental amalgam were prepared by using the below method:

1. Metallic silver (Ag) and tin (Sn) with high purity were alloyed forming a homogeneous alloy of silver and tin. The formed silver-tin alloy was then reduced to form a fine powder. The fine silver-tin powder was then reacted with hydrochloric acid to remove the tin from the alloy thereby leaving solid particles of treated silver in the acid solution. The process of hydrochloric acid treatment were carried out several times on the solid silver-tin alloy particles until there was a noticeable color change in the solid particles from a dark grey to a yellowish brown color. The yellowish brown solid particles were filtered and washed several times with warm water to remove all traces of the acid and the particles were dried in an oven to form the treated silver composition.
2. The silver-mercury (Ag_3Hg_2) compound was prepared by reacting the silver granules and mercury separately with nitric acid to form silver nitrate and mercury nitrate solutions. The two solutions were subsequently mixed and diluted by adding water. Metallic copper of high purity was placed in the mixture to precipitate the silver mercury composition. The precipitated silver-mercury composition was filtered using filter paper and washed several times with warm water to remove traces of acid and then dried in an oven.
3. To form the dental amalgam, 65 % of treated silver and 35 % of silver-mercury were mixed to form a compound powder. The mixed compound powder was then

combined with mercury in the ratio of 1:1.62 and then triturated using high speed amalgamator (Ultramat 2, SDI, Australia) for 3 s forming an amalgam paste.

All the starting materials and Silverfil amalgam were characterized closely using FESEM-EDX, XRD and XPS.

2.2 Characterization of Samples

X-Ray Photoelectron Spectroscopy (XPS) (Kratos AXIS Ultra 'DLD', Manchester, United Kingdom) was used to provide information on the chemical state of the materials. The analysis was performed in Ultra High Vacuum (UHV) at 6.66×10^{-8} Pa. The high-resolution of narrow scan spectra were obtained with an analyzer pass energy of 20 eV. The binding energy was calibrated using the C 1 s peak of 285.0 eV from adventitious carbon. The crystal phase and structure of the materials were determined by X-Ray Powder Diffraction (XRD) (D8 Advance diffractometer, Bruker, Germany) with graphite secondary monochromatized Cu-K α radiation at 40 kV and 40 mA. Data collection and manipulation using EVA software which contains a data base of the Joint Committee for Powder Diffraction Standard (JCPDS) powder diffraction files. The pattern was obtained in the range of 2–80°, with a 2θ step scan of 0.02°. The morphology of the amalgam was observed through Field Emission Scanning Electron Microscopy (FESEM) (Quanta 200, FEI, Hillboro, USA) and the elemental composition of the samples were analyzed using Energy Dispersive X-ray Spectroscopy (EDX) equipped in SEM.

3 Results and Discussion

Silverfil amalgam was synthesized by amalgamation of a mixture powder of treated silver particles and silver-mercury alloy powder with mercury. The following chemical reaction shows the process of amalgamation of Silverfil:



Sample identification:

Sample A: Ag.

Sample B: Ag₃Hg₂.

Sample C: Ag₂Hg₃ + 2Ag.

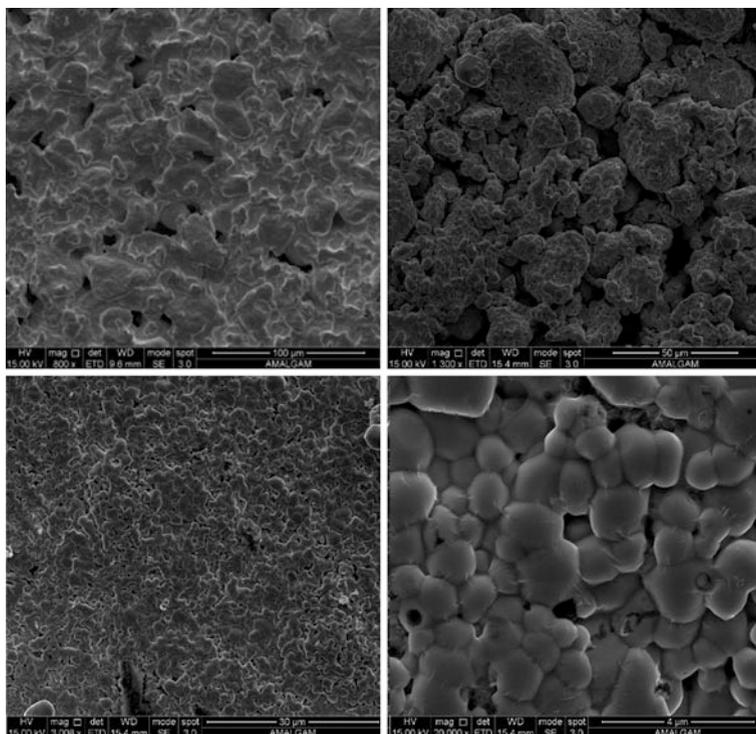


Fig. 1 SEM micrograph of Silverfil amalgam surface in different magnifications

3.1 SEM and EDX Energy Dispersive X-Ray Spectroscopy Analysis

SEM and EDX were used for microstructure morphology and elemental composition. Figure 1 shows the SEM structure of Silverfil amalgam (sample C). The silver-mercury clusters were formed as a crystal matrix compound. Mercury dissolves the treated silver to form intermetallic compound, Ag_2Hg_3 and the crystals grow as a particle binding together with the excess silver.

The chemical composition of treated silver (sample A), silver-mercury (sample B) and Silverfil amalgam (sample C) were obtained using SEM-EDX are represented in Figs. 2, 3, 4.

The EDX spectrum in Fig. 2 shows that the treated silver consists of the pure Ag element with weight percentage of 100 %. Sample B (Fig. 3) consists of only Ag and Hg with weight percentage of 38.17 and 61.83 % relatively. These samples were relatively pure because they do not contain any impurities. Figure 4 shows the elemental composition of Silverfil amalgam consisting of Ag and Hg with weight percentages of 28.22 and 71.78 %, respectively.

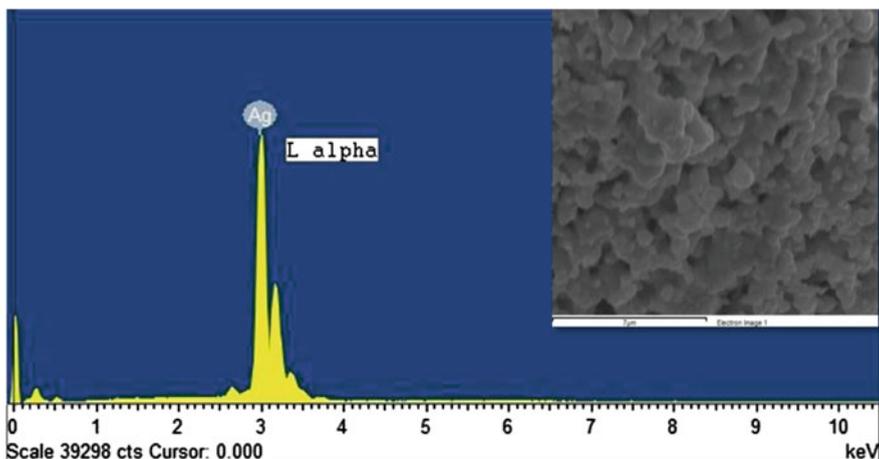


Fig. 2 SEM-EDX analysis of treated silver (sample A)

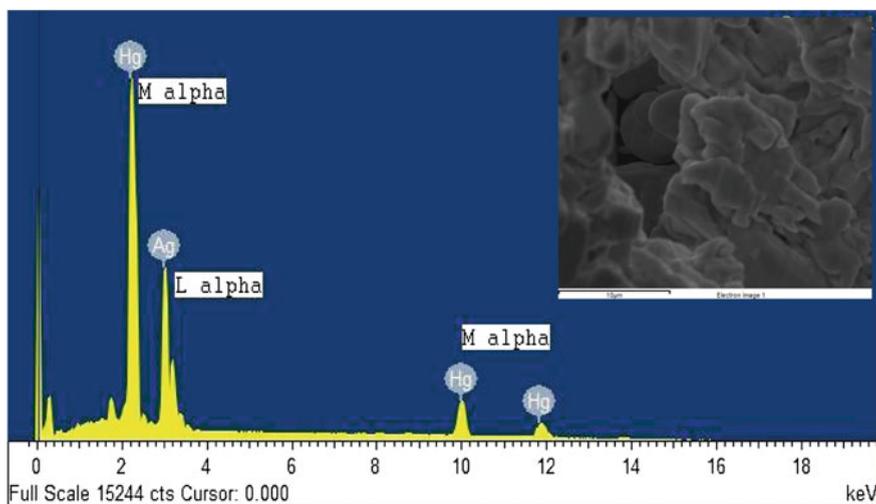


Fig. 3 SEM-EDX analysis of silver-mercury (sample B)

3.2 Crystalline Studies by XRD Analysis

The crystallinity and the phase purity of the starting materials and Silverfil amalgam were further characterized by XRD. All of the diffraction peaks from samples were compared with the JCPDS file. Figure 5 shows the XRD pattern of treated silver. The pattern shows that the samples are crystalline and the position (interplanar d_{hkl}) and relative intensities (normalized with respect to maximum value 100) in the peaks compare well the value reported in data base [17] which

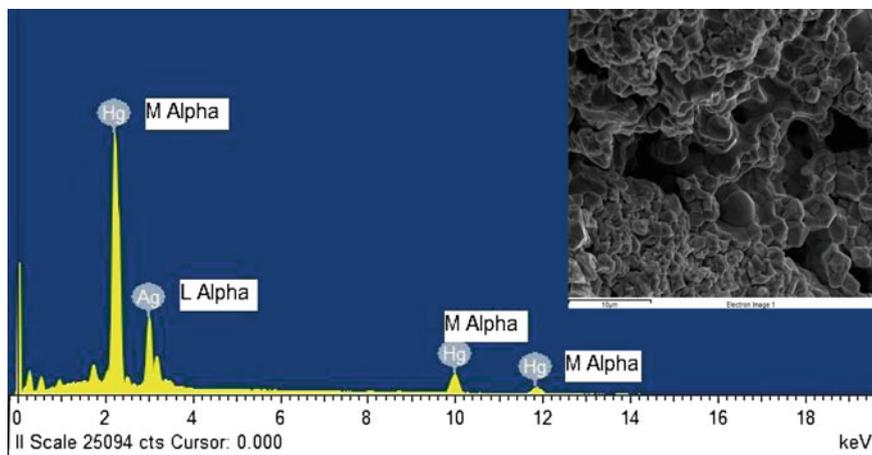


Fig. 4 SEM-EDX analysis of Silverfil amalgam (sample C)

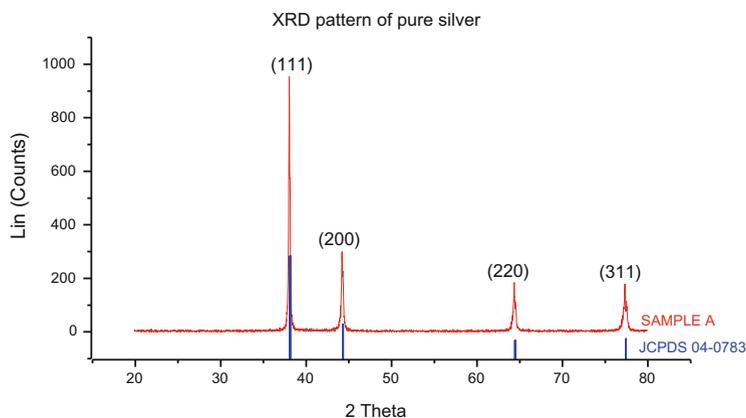


Fig. 5 XRD pattern of pure treated silver (sample A)

indices pure silver. The diffraction peaks originated from (111), (200), (220) and (311) planes of pure silver and pack as cubic. All the diffraction peaks are sharp with (111) diffraction peak having the highest intensity. This result agrees well with the SEM-EDX analysis.

Figure 6 compare well (interplanar spacing d_{hkl} and relative intensities in the peaks) with the data base [18] suggesting the sample is a crystalline silver-mercury structure with the composition of Ag_2Hg_3 and also Ag_3Hg_2 [19]. The XRD pattern of Silverfil amalgam (sample C) is shown in Fig. 7. The diffractogram shows that the sample is in the form of crystalline. All the diffraction peaks of this sample with (411) peak having the highest intensity could be readily indexed the value reported in data base of XRD [20]. This suggested that Silverfil amalgam is in the

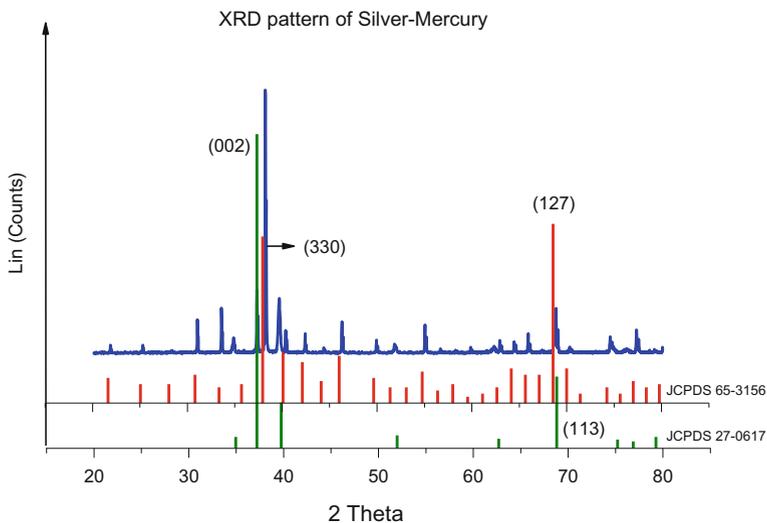


Fig. 6 XRD pattern of silver-mercury (sample B)

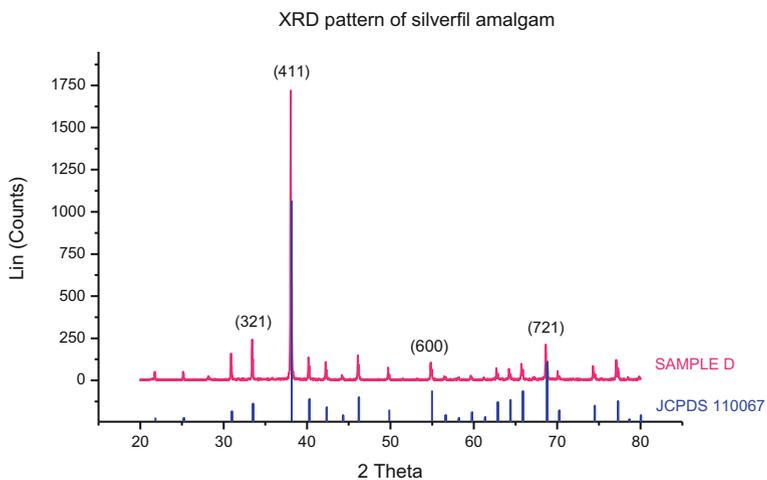


Fig. 7 XRD pattern of Silverfil amalgam (sample C)

form of isometric (cubic)-hexaoctahedral with a unit cell of 10.06 \AA which is similar to gamma moschellandsbergite, a natural mineral [20]. All the peaks of Silverfil amalgam are similar to the gamma-moschellandsbergite which has the molecular formula Ag_2Hg_3 . This infers that Silverfil is safe to be used and stable because of the fraction of stable compounds as Ag_2Hg_3 .

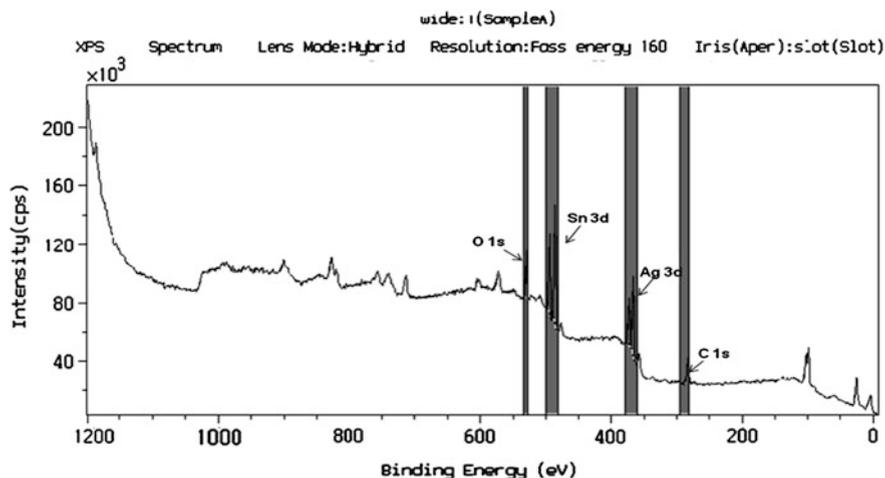


Fig. 8 Wide scan of XPS spectrum of treated silver (sample A)

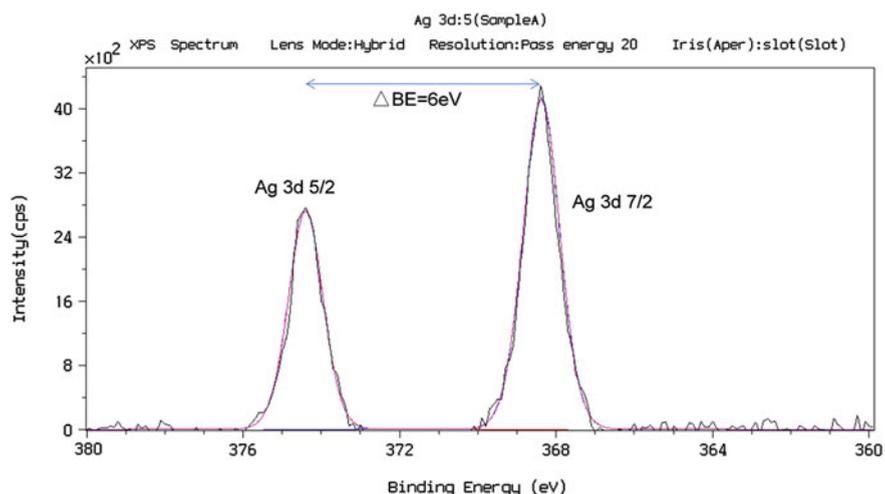


Fig. 9 Narrow scan of XPS spectrum of Ag 3d for treated silver (sample A)

3.3 Surface Analysis using XPS Technique

The position of photoelectron peak as binding energy (eV) can be confirmed by comparing the position of the photoelectron peak with the data base value in the XPS handbook. Figure 8 shows the wide scan of XPS spectrum of treated silver (sample A). The spectra contained expected photoelectron peaks of O 1s with relative mass concentration 18.45 %, Ag 3d, 26.27 %, Sn 3d, 43.69 % and C 1s,

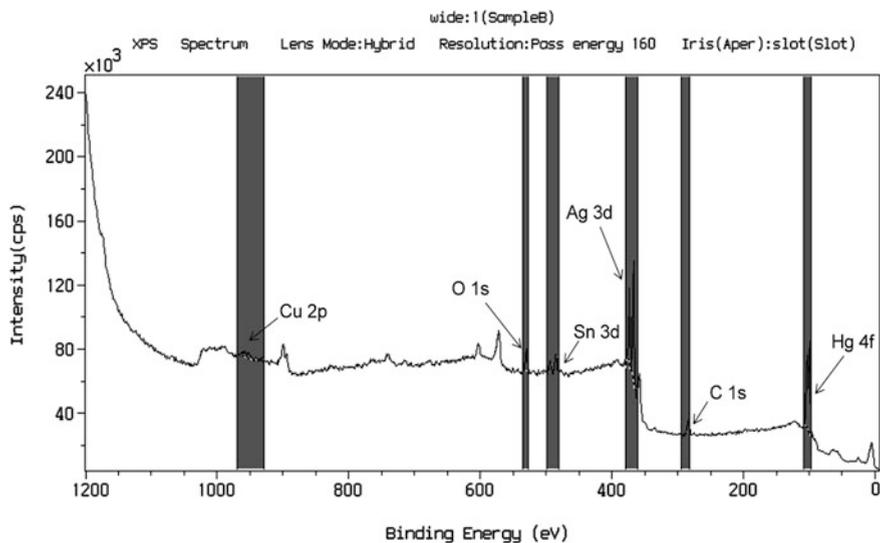


Fig. 10 Wide scan of XPS spectrum of silver-mercury (sample B)

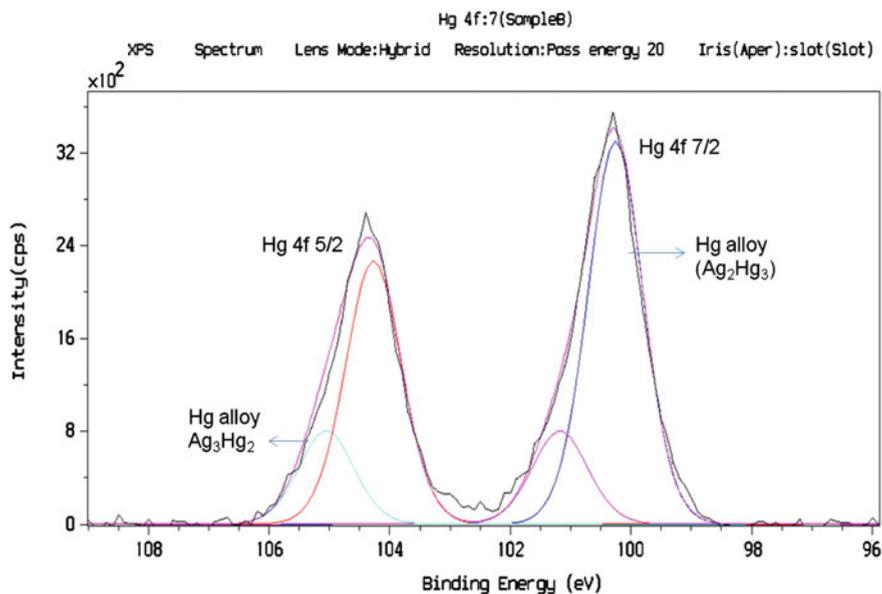


Fig. 11 Narrow scan of XPS spectrum of Hg 4f for silver-mercury (sample B)

11.59 %. Tin is observed as impurities during the preparation of treated silver. The narrow scan of Ag 3d of sample A (Fig. 9) shows the formation of Ag^0 with the binding energy of 368 eV [21]. The line were deconvoluted into synthetic peaks in relation $I(\text{Ag } 3d_{5/2})/I(\text{Ag } 3d_{3/2}) = 3/2$ [16].

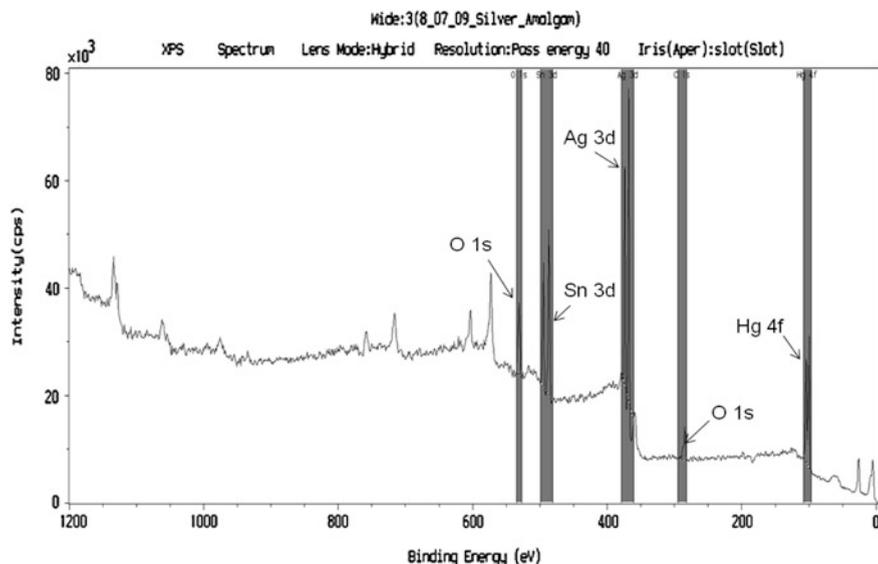


Fig. 12 Wide scan of XPS spectrum of Silverfil amalgam (sample C)

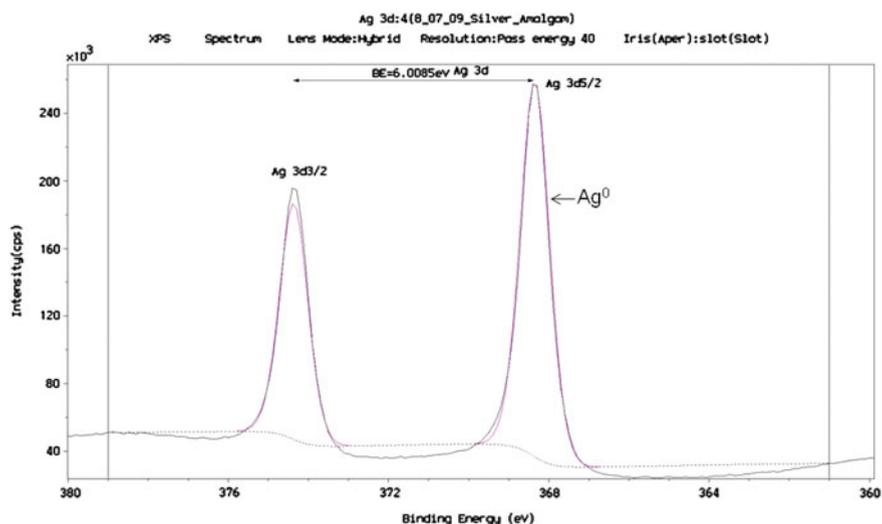


Fig. 13 Narrow scan of XPS spectrum of Ag 3d for Silverfil amalgam (sample C)

Figure 10 shows the wide scan of XPS spectrum for silver-mercury (sample B) with mass concentration of Ag 3d 31.29 % and Hg 4f 47.82 %. Other elements like Cu, Sn, O and C appear in a small percentage (less than 8 %). Figure 11

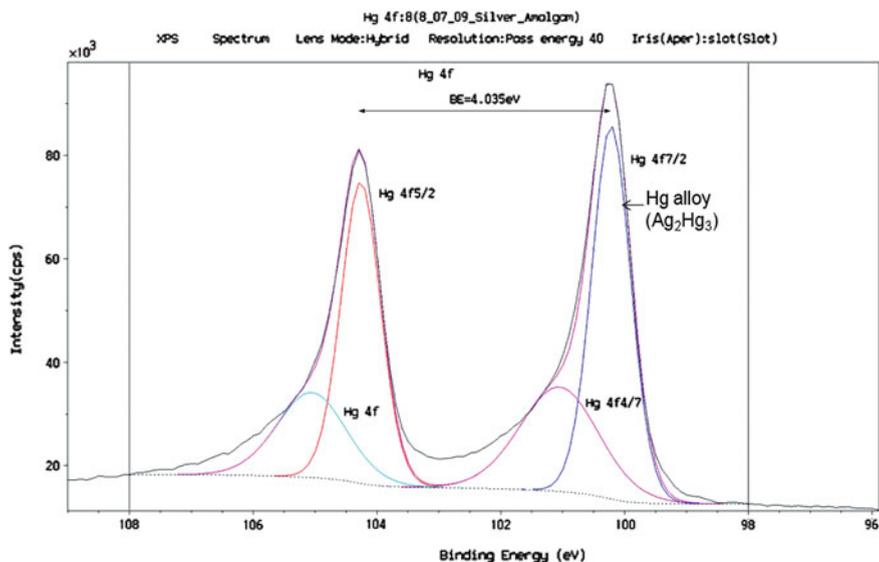


Fig. 14 Narrow scan of XPS spectrum of Hg 4f for Silverfil amalgam (sample C)

shows the narrow scan of mercury for sample B. The peaks in Fig. 11 show the value of binding energy 101.1 eV (Hg 4f_{5/2}) and 104.2 eV (Hg 4f_{7/2}). This binding energy corresponds to Hg alloy [21].

Figure 12 shows the wide scan of XPS spectrum of Silverfil amalgam (sample C). The spectra contain expected photoelectron peaks of O 1s at 531.2 eV, C 1s at 286.14 eV, Sn 3d at 495.32 eV, Hg 4f at 104.25 eV and Ag 3d at 368.0 eV.

Narrow scan of Ag 3d (Fig. 13) shows doublet pair which arises due to spin-orbital coupling 3d_{5/2} and 3d_{3/2}. The binding energy for the Ag 3d_{5/2} and Ag 3d_{3/2} peaks are 368.0 eV and 374.0 eV respectively. The difference between the two binding energy is 6.0 eV. These peaks at this specific binding energy value confirm the formation of silver as Ag⁰.

The binding energy for Hg 4f (Fig. 14) shows peaks Hg 4f_{5/2} at 104.2 eV and Hg 4f_{7/2} at 100.2 eV. The difference in binding energy of 4.0 eV corresponds to Hg alloy [16]. This confirms that the surface study of Silverfil amalgam has proven that the sample does not contain any free mercury, Hg⁰.

4 Conclusion

In conclusion, the surface studies showed that the treated silver is a porous material which can absorb mercury and bind together. This has been proven through XPS analysis where studies have clearly shown that the mercury used for

the amalgamation process completely reacts with treated silver to form Ag_2Hg_3 and there is no pure mercury in the filling material. XRD analysis also confirmed that the final Silverfil amalgam is similar to a mineral in nature called “Moschellandsbergite” which is formed as Ag_2Hg_3 .

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An *in vitro* Study of Genotoxicity of Silver Amalgam

Akram Hassan, Muhammad Hidayat Muhamad Daud, Zaihan Ariffin,
Mohammad Khursheed Alam

ABSTRACT

Background: Silver amalgam/Silverfil Argentum® is a 'Malaysian made amalgam' has already been approved to be free from cytotoxicity, however its genotoxic effect has not been explored yet as biocompatible material. The objective of this study was to identify the genotoxic characteristic of silver amalgam by using Bacterial Reverse Mutation Assay (Ames test).

Materials and Methods: This was a descriptive experimental study involving two strains of mutated *Salmonella*. The test material was evaluated in two mutated strains of *Salmonella typhimurium* TA98 and TA100 with and without an external metabolic activation system (S9 Mix). The bacteria were incubated for 48 hours at 37 ± 0.5 before the colony growth or revertant colonies were counted. Data obtained were analyzed by using non-statistical method.

Results: The investigation of the genotoxic reaction on the test material revealed that the numbers of revertant colonies in both strains with and without S9 Mix were less than twice of the negative control even in the presence of high silver amalgam concentrations (5.0 µg/ml).

Conclusion: This study demonstrated that the test material did not exhibit any mutagenic activity under the chosen conditions. Thus, silver amalgam could be considered to have no genotoxicity effect.

KEY WORDS

silver amalgam, genotoxicity, Ames test

INTRODUCTION

Amalgam was recognized to be the most ancient and long lasting restorative material ever been made until now. In spite of good hardness properties, level of toxicity of mercury content is keep declining over the years. Many companies of amalgam product passionately running in research and put full effort in order to notify their product to be free from mutagenesis. Silverfil Argentum® is a Malaysian made amalgam (Mohamad *et al.* 2013) developed since 10 years ago has successfully received FDA 510 (K) clearance from the United States Department of Health and Services in America.

An international standard (ISO) lays down specific requirements for biocompatibility, including the tests based on the nature of the contact and the duration of implantation of the biomaterial. The standard stipulates that all materials that will be in contact with mucous membrane, bone or dentinal tissue where the contact exceeds 30 days, as well as all implantable devices where the contact exceeds 24 hours, must undergo genotoxicity testing (Chauvel-Lebret *et al.*, 2001). According to International Organization for Standardization (ISO) (1992), when the genetic toxicity of a medical device has to be experimentally assessed, a series of *in vitro* tests shall be used, this series shall include at least three assays and at least two of these should preferably use mammalian cells as a target.

Kirkpatrick *et al.* (1997) and Katzer (2002) suggested to use an *in vitro* study after allowing for cost intensive and time consuming. Moltelmans and Zeiger (2000) strongly agreed that *Salmonella typhimurium* / microsome assay to be widely accepted as a short-term bacterial assay in order to identify the substance that can produce genetic damage that leads to gene mutations. To reduce the number of biomechanical studies, evaluation of new biomaterials should nowadays

begin with *in vitro* cytotoxicity and mutagenicity tests. This applies for the development of both temporary and permanent implants and prostheses and for permanent implant particularly under the aspect that carcinogenic potential is often the consequence of chronic exposure to minute concentrations (Katzer *et al.*, 2002). Although Silverfil Argentum® had successfully explored in cytotoxic effect studies, the genotoxic characteristics have to be carried out before it can be known as biocompatible material.

Therefore, the aim of this study was to identify the effect of genotoxicity in this product.

MATERIALS AND METHODS

Test Material

Silverfil Argentum® which obtained from Dunia Perwira Manufacturing Sdn. Bhd. in capsule form was triturated by using amalgamator for 5 seconds. The bolus was let to be dried and hardened before it can be powderized. The powder was weighted into 5 unit of scale of 5000 µg/2500 µg/1250 µg/625 µg/313 µg per plate respectively by using micro weight machine (mg scale). Each unit scale of powder was then diluted into 1 ml of Dymethylsulfoxide (DMSO) solution and kept inside incubator for 24 hours at 37 ± 0.5 rolling prior to the procedure. Apart of it, 60% reactive silver and 40% silver mercury makes Silverfil Argentum® less expose into cytotoxic effect. It saved inside autoclave machine with 121 heated as the boiling point reaching 357 .

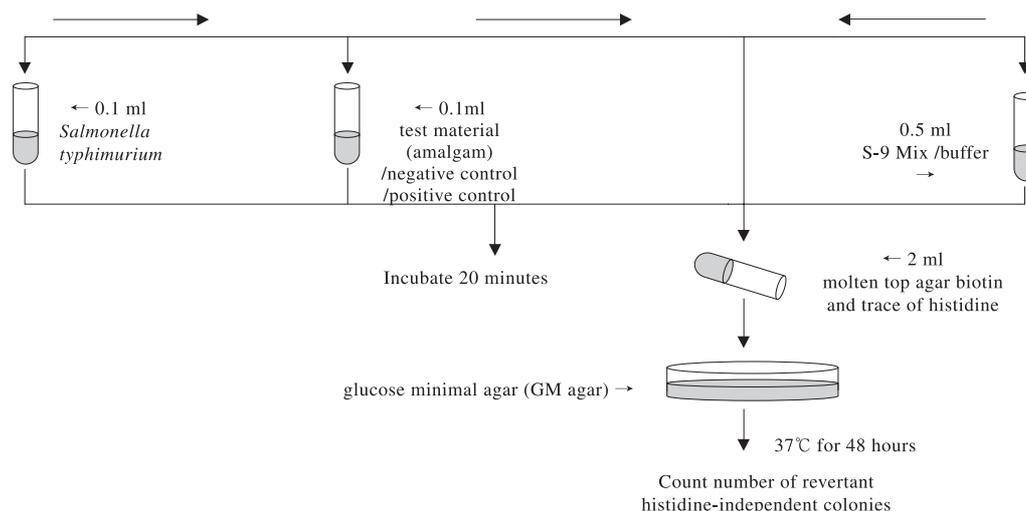


Figure 1. Overview of bacterial reverse mutation test (Ames Test) process

Table 1. Positive Controls

Strain	Positive control chemical (-S9)	Molar concentration (mg/plate) ^a	Positive control chemical (+S9)	Molar concentration (mg/plate) ^a
TA98	4-Nitro-o-phenylenediamine	2.5	2-Aminoantracene	2.5
TA100	Sodium Azide	5.0	2-Aminoantracene	2.5

a - Concentration based on 100 x 15 mm petri plate containing 20 to 25 ml of GM agar (Mortelmans and Zeiger, 2000).

Ames Test

Ames test is a method introduced by people named B. Ames, J. Mc Cann and E. Yamasaki in 1975 using special genotype variants of the bacterium *Salmonella typhimurium* (Katzer, 2001). Basically, the bacteria was mutated and histidine dependent (lack of histidine genes) which unable to grow onto histidine-free agar. Once being exposed to the mutagens, it will revert and become histidine independent that make it able to grow and form a colony on the same plate. Here, TA98 and TA100 were selected strains used under samples 471 approved by International Organization for Standardization (1992). Genetically, TA98 is going to be for detection of frameshift mutation while TA100 is responsible on base-pair substitution mutation (Chang, 2002).

Therefore, reviving these two strains before use are very important particularly for genetic analysis. Those strains were obtained from Dr. T. Nohmi from National Institute of Health Science, Tokyo, Japan and were stored as frozen stock cultures (0.035 ml of DMSO/0.4 ml of broth culture) at -80 ± 5 in ultra-deep freezer (MDF 392AT, SANYO).

In this study, metabolic activation system was practically used to activate the Silverfil Argentum® to be more mutagenic, thus increase the number of colony growth on the agar. It contained cytochrome-based P450 mimicked endogenous human liver. An exogenous mammalian organ activation system that consists of a 9000xg supernatant fraction of a rat liver homogenate (S9) with presence of cofactors for NADPH-supported oxidation was used (Mortelmans and Zeiger, 2000).

Sterile distilled water was used as negative control (Chang, 2002). Positive control chemicals were specifically designed as highly mutagenic to each strain. 4-Nitro-o-phenylenediamine (4-NoPD) was definitely for strain TA98 and Sodium azide (NaN3) was specifically for strain TA100. Both were applied without S9 (replaced with Sodium phosphate buffer). In another hand, 2-Aminoantracene (2AA) was used in S9 inclusion (Mortelman and Zeiger, 2000).

S9 mix and buffer were obtained from Kikkoman Co. Ltd. and stored inside ultra-deep freezer under -80 ± 5 (MDF 392AT,

SANYO).

Reviving (Frozen Working Cultures)

1 ml of *Salmonella typhimurium* was inoculated into 5 ml of nutrient broth and nutrient agar. This procedure was aseptically done in a 'Lamina Flow'. These two mediums were then incubated for overnight in the incubator (SANYO) at 37 ± 0.5 . The result was shown cloudy look in nutrient broth and colonies were formed on the nutrient agar. One isolated healthy colony was transferred onto Glucose Minimal (GM) agar and incubated for 48 h at 37 ± 0.5 . This would later produced colony and subculture was repeated for another 48 hours. The last colony formed on the GM agar so-called master plate was being run for genetic analysis by streaking the bacteria onto histidine, biotin and histidine-biotin filled agar for 15 hours (Mortelmans and Zeiger 2000, Chang 2002). There were no growths seen in histidine and biotin agar but presence on histidine-biotin agar, rfa marker and presence of plasmid pKM101 with ampicillin resistance had shown growth inhibition which proved that TA98 and TA100 were mutated on specific genotype.

Main Test

After preparing frozen working cultures which underwent genetic analysis, TA98 and TA100 were ready to be used. Silverfil Argentum® was incubated at 37 ± 0.5 for 24 hours prior to the procedure. Amount of S9 fraction was measured within the range 10-30% (v/v) in the S9 mix by using the formula calculated below:-

$$10\% = \frac{0.04 \text{ ml S9 to } 0.1 \text{ ml S9}}{1 \text{ ml cofactor per plate}}$$

So, 10% (v/v) was used for this test and was ready inside sterile glass tube containing ice cube and maintained cold along the procedure.

For the 13 x 100 mm sterile glass tube, the following orders were

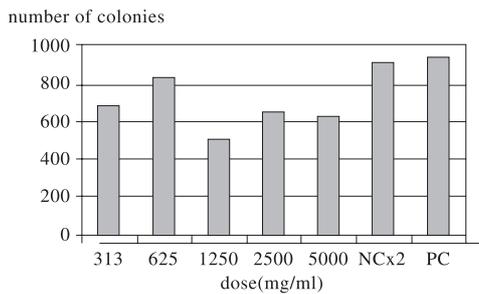
Table 2. Result for *Salmonella typhimurium* TA98

Substance		Number of revertant colonies		
Negative control		455		
Positive control		932		
Test substance(µg/plate)	Plate 1	Plate 2	Mean	
without	313	483	880	682
S9 Mix	625	683	1001	842
	1250	432	584	508
	2500	296	1017	657
	5000	669	584	627
	Negative control		125	
Positive control		298		
with	Test substance(µg/plate)	Plate 1	Plate 2	Mean
S9 Mix	313	232	139	186
	625	156	155	156
	1250	125	168	147
	2500	203	104	154
	5000	137	143	140

Table 3. Result for *Salmonella typhimurium* TA100

Substance		Number of revertant colonies		
Negative control		321		
Positive control		791		
Test substance(µg/plate)	Plate 1	Plate 2	Mean	
without	313	69	109	89
S9 Mix	625	593	277	435
	1250	541	130	336
	2500	101	85	93
	5000	135	142	139
	Negative control		410	
Positive control		858		
with	Test substance(µg/plate)	Plate 1	Plate 2	Mean
S9 Mix	313	997	275	636
	625	804	61	433
	1250	96	99	98
	2500	658	230	444
	5000	264	460	362

TA98



TA100

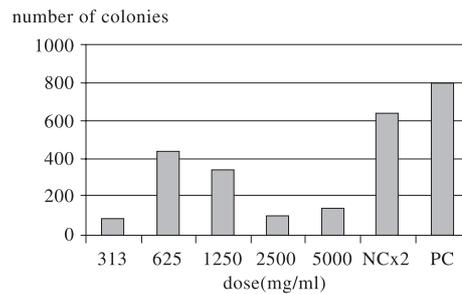
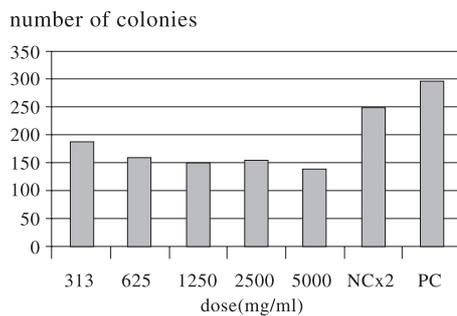


Figure 2. Tests in two strains without metabolic activation (-S9 Mix)

TA98



TA100

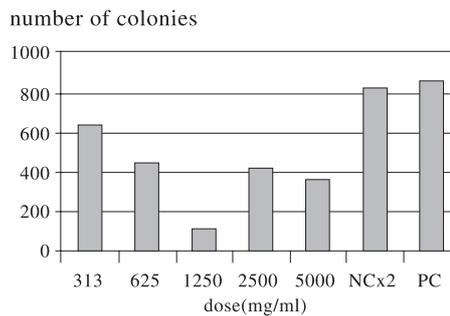


Figure 3. Tests in two strains with metabolic activation (+S9 Mix)

added with mild mixed:

- * 0.1 ml Silverfil Argentum® extracts
- * 0.1 ml *Salmonella* strain
- * 0.5 ml sodium phosphate buffer, 0.1 M pH 7.4

The mixture was incubated for 20 minutes at 37 ± 0.5 . Then, 2 ml of molten top agar was added, gently mixed and quickly poured onto the surface of GM plate agar. In order to make sure of evenly

distributed, this procedure was done within 2 minutes and manually stirred. After the top agar was hardened, the plate was incubated in inverted position at human temperature for 48 hours. Final result was obtained by counting the colony growth using Colony Analyzer (ProtoCol.). The procedure was standardized for S9 mix, solvent and positive controls.

Amount of positive control chemicals were calculated by using a formula gained below:-

$$M1V1 = M2V2 \text{ (M = molar, V = volume)}$$

$$M1 = \text{molar concentration (x)} \quad M2 = \text{molar concentration of chemical}$$

$$V2 = 0.7 \text{ ml solution} \quad V1 = 0.1 \text{ ml solution}$$

No ethical clearance needed for this test seems it was not involved human and animal product.

Statistical Consideration

This approach also has its weaknesses. It has been shown that the two-fold rule may be too insensitive for *Salmonella* strains with relatively high reversion frequencies, such as TA100, TA97, and TA102, and too sensitive for chemicals with low reversion frequencies, such as TA1535 and TA1537. Therefore, non-statistical approach was applied here. Mortelmans and Zeiger (2000) implemented following criteria to interpret results.

Positive: A compound is considered a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains.

Negative: A compound is considered a nonmutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments.

Inconclusive: If a compound cannot be identified clearly as a mutagen or a nonmutagen, the results are classified as inconclusive.

RESULTS

The test substance is considered to be mutagenic when the number of counted colonies exceeds the number of colonies in the negative controls by at least double and a relationship between dose and response can be observed. The results in the *Salmonella* tester strains are presented in Table 2 and 3 and shown in Figure 2 and 3.

The results showed the numbers of revertant colonies which were treated with the test substance were less than twice of negative control with and without S9 Mix at all concentrations. No dose-response relationship was obtained and no statistical procedure was used.

The results showed the numbers of revertant colonies which were treated with the test substance were less than twice of negative control with and without S9 Mix at all concentrations. No dose-response relationship was obtained and no statistical procedure was used.

Figure 2 showed that the test for mutagenesis in two strains without metabolic activation (-S9 Mix) with different concentrations of Silverfil Argentum®, gave no evidence for mutagenic effects.

Figure 3 showed that the test for mutagenesis in two strains with metabolic activation (+S9 Mix) with different concentrations of Silverfil Argentum®, gave no evidence for mutagenic effects.

DISCUSSION

Based on International Standard, ISO 10993-2 (1992), approximately eight different *in vitro* assays are listed down for researchers to choose. However, the best two tests would be Organization for Economic Cooperation and Development (OECD) tests which were *Salmonella typhimurium* reverse mutation assay (471) and mammalian cytogenetic test (473). In this study, the assay used was *Salmonella typhimurium* reverse mutation alone and it showed very significant findings. Margolin et al. (1985) mentioned of using TA98 and TA100 as a two out of 6 recommended strains of bacteria with and without metabolic activation (S9) system in this test was sufficient to identify approximately 90% of the mutagens in a population containing about 35% mutagenic chemicals.

Although all known assays can yield false-positive and false-negative results, experience shows that the combination of two different test methods is a reliable parameter for determining carcinogens which are a risk to human health (Katzner et al., 2002).

A minimum of two assays is needed as a battery that is capable of detecting most potential mutagens and genotoxic carcinogens. And this study is only a part of an *in vitro* evaluation of genotoxicity of Silverfil Argentum. Concentrations of Silverfil Argentum® with a range from 313 µg to 5000 µg were chosen as standard values given

by Mortelmans and Zeiger (2000) because this material has previously approved to be out of cytotoxic effect and yet the 5000µg was the highest indicator value to respond to any genotoxic activity.

To ensure the comparability of results, the extraction temperature should preferably be 37 ± 0.5 that mimicked the human body temperature and the time must be at least 24 hours. Silverfil Argentum® was dissolved into distilled water and not DMSO simply because DMSO solution is known to be high cytotoxic. This study involves the mixture of S9 as metabolic activation system because *Salmonella typhimurium* is only a prokaryote (basic cell living structure) and cannot represent the human being as a perfect model; therefore the addition of the exogenous liver enzyme could enhance the quality of the result.

After 48 hours incubated, the visible colonies were only *Salmonella typhimurium* and not other organisms. It was due to highly mutated characteristic of *Salmonella typhimurium* both at the level of gene transcription and gene translation that had been proven through genetic analysis. This would make the bacteria to be strong enough to kill other organisms and able to grow in the absence of histidine plate agar. The revertants were counted by using Colony Analyzer (ProtoCol.). The specificity and sensitivity was fixed to 96% with 8cm diameter of counter area. This measurement homogeneously has been done to all plates.

From the data, we found that Standard Deviation (SD) value generally was high and it was due to small samples and replications. However, if we deal with experimental environment, it is enough to only duplicate or triplicate the test if the first trial gives a set of data within expected result. Nevertheless, in the future *in vitro* genotoxicity test which will preferably use more strains and mammalian cells as target should be carried out. *In vivo* genotoxicity test can be undertaken if scientifically indicated or *in vitro* test results indicate potential genotoxicity. Technique sensitive and low sterility were the main problems here and need to be overcome in order to get better results. A nanotechnology measurement technique hopefully can be applied to cut the tester material into more identical size according to reference doses.

CONCLUSION

These data were demonstrating as negative result and showing the test material Silverfil Argentum did not exhibit mutagenic activity and no dose-related increase in the number of revertant colonies under the present test conditions. Thus, the material was considered to have no genotoxic effect.

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