BIOSERVICE

SCIENTIFIC LABORATORIES GmbH

In vitro Cytotoxicity Assay:

Cell Growth Analysis via BCA-Staining with an Extract of Surgical stainless steel tray coated with NanoMoldRelease coating

Report

BSL BIOSERVICE Project No.: 074050

Sponsor:

Nanoplas, Inc.

2950 Prairie St., SW,

Suite 900

Grand Rapids, MI

49418 USA

RZOODE77, IBAN: DE31 7402 (
IBAN: DE52 7007 0024 094C

Copy of the GLP-Certificate

BAYERISCHES LANDESAMT
FUR ARBEITSSCHUTZ,
ARBEITSMEDIZIN UNO SICHERHEITSTECHNIK

Pfarrstrali>e 3 · 80538 MUnchen ·Telefon (089) 21 84-0

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GLP-Bescheinigung/ Statement of GLP Compliance

{gema f),/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Oberwachung der Einhaltung der GLP-Grundsatze gernaf1 Chernikaliengesetz bzw. Ricl1tlinie 881320/EG wurde durchgefOhrt in:

Assessment of conformity with GLP according to Chernikaliengesetz and Directive 88/320/EEC at:

PrUfeinrichtung/Test facility D PrOfstandort/Test site

BSL Bioservice Scientific Laboratories GmbH
Behringstrasse 6
82152 Planegg
(Unverwechse!bare Bezeichnung und Adresse/Unnquivocal name cind address)

Prufungen nach Kategorien/Areas of Expertise
(gemillllaccording ChernVwV-GLP Nr. 5.3/0ECD guidance)

2 PrOfungen auf toxikologische Eigenschaften
3 PrOfungen auf mutagene Eigenschaften (in vitro/in vivo)
9 Sonstige PrOfungen:
a) Mikrobiologische SicherheitsprOfungen
b) WirksamkeitsprOfungen an Zellkulturen

Datum der lnspektion/Date of Inspection
(Tag.Monat..lahr!day.month.year)
11./12.02.2004

Die/Der genannte PrOfeinrichtung/PrOfstandort befindet sich im nationalen GLP-Oberwachungsverfahren und wird regelrnal),ig auf Einhaltung der GLP..Grundsatze Uberwacht.

Tt1e above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a rngular basis.

f\uf der Grundlage des lnspektionsberichtes wird hiermit bestatigt, dass in dieser PrOfeinrichtung/ cliesern PrOfstandort die oben genannten PrOfungen unter Einhaltung der GLP-Grundsatze durchgefUhrt we en konnen.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

//4 Munchen, 21.07.2004

I.V.
Ritter U- -

Leitender Gewerbedirektor

L.S.

Contents

Preface

General

Sponsor:	**Nanoplas, Inc.** 2950 Prairie St., SW Grandville, MI 49418 95,
Test Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstrasse 6 82152 Planegg Gennany
BSL BIOSERVICE Project-No.:	074050
Test Item:	Surgical stainless steel tray coated with NanoMoldRelease coating
Title:	*In vitro* Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of Surgical stainless steel tray coated with NanoMoldRelease coating

Project Staff

Study Director:	M. Sc. Sonja Helmich
Deputy Study Director:	Dipl.-Ing. (FH) Kristin Rodig
Management:	Dr. Wolfram Riedel Dr. Angela Lutterbach
Quality Assurance Unit:	Dipl.-Biol. Uwe Hamann Dr. Margarete Hoechst Dr. Helga Kohn Gwendolyn Pretzsch, B.A. Dip!. oec. troph. Ulrike Fuhnnam1

Schedule

Anival of the Test Item:	November 13, 2007
Date of Draft Project Protocol:	November 21, 2007
Date of Project Protocol:	November 23, 2007
Start of Experiment:	November 26, 2007
End of Experiment:	November 30, 2007
Date of Report:	December 18, 2007

Project Staff Signatures

Study Director: M. Sc. Sonja Helmich

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Date: Dec 18, 2007

Management:

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Date: .....

Quality Assurance

CLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Gern1any, Appendix 1 to §19a as amended and promulgated on June 20, 2002 (BGB 1. I Nr. 40 S. 2090), revised October 31, 2006 (BGB 1. I Nr. 50 S. 2407).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study was assessed in compliance with the project protocol, the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and/or the test facility are periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections and audits are included in the report. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of the staff involved in the study. The final report of the study was audited. A Quality Assurance Statement, signed by the Quality Assurance Unit, is included in the report.

The test method is part of the BSL BIOSERVICE accreditation scope according to guideline 901385/EWG, 93/42/EWG and DIN EN ISO/IEC 17025 for testing of medical devices.

Guidelines

This study follows the procedures indicated by the following internationally accepted guidelines:

Biological evaluation of medical devices:

ISO 10993-1: 2003, ,,Evaluation and testing"

ISO 10993-5: 1999, ,,Tests for *in vitro* cytotoxicity"

ISO 10993-12: 2002, ,, Sample preparation and reference materials"

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to GLP-regulations:

A copy of the final report, the project protocol, the study plan and a documentation of all raw data generated during the conduct of the study (documentation fom1s as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the project.

If test item is left over a sample will be stored according to the period fixed by the GLP-regulations. Samples that are unstable may be disposed of before that time.

Unless otherwise agreed upon, remaining test item will be discarded three months after release of the report.

Statement of Compliance

BSL BIOSERVICE Project-No.:	074050
Test Item:	Surgical stainless steel tray coated with NanoMoldRelease coating
Title:	*In vitro* Cytotoxicity Assay: Cell Growth Analysis via SCA-Staining with an Extract of Surgical stainless steel tray coated with NanoMoldRelease coating
Study Director:	M. Sc. Sonja Helmich

This study performed in the test facility BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Gennany, Appendix I to §19a as amended and promulgated on June 20, 2002 (BGBI. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407).

"OECD Principles of Good Laboratory Practice", as revised in 1997, Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: M. Sc. Sonja Helmich

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Date: Jan. 02. 2008

Statement of the Quality Assurance Unit

BSL BIOSERVICE
Project-No.: 074050

Test Item: Surgical stainless steel tray coated with NanoMoldRelease coating

Title: *In vitro* Cytotoxicity Assay: Cell Growth Analysis via BCA-Staining with an Extract of Surgical stainless steel tray coated with NanoMouldRelease coating

Study Director: M. Sc. Sonja Helmich

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

Audit	**Dates of QAU Inspections**	**Dates of Reports to the Study Director and Management**
Project Protocol I Study Plan	November 23, 2007	November 23, 2007
Experimental Phase (Method Audit)	October 16, 2007	October 16, 2007
Report	December 28, 2007	December 28, 2007

This report reflects the raw data.

Member of the
Quality Assurance Unit:

Date: .:'.-::..../.8.:...,.':.:'...:'(C

Summary

In the present study the cytotoxic effects of Surgical stainless steel tray coated with NanoMoldRelease coating were analysed. Hereby, the test item was extracted under agitation for 22 h with cell culture medium and the extract was incubated with L929 cells for 69.25 h. The protein content of the individual cultures was then analysed as a measure for cytotoxicity and compared to those of the controls.

In this study under the given conditions no leachable materials were released in cytotoxic concentrations from the test item.

Introduction

Cytotoxicity tests represent one of the easiest methods for the analysis of detrimental effects of substances. Cell culture techniques allow a rapid yet sensitive diagnosis of the biological reactivity of leachable or diffusable components of materials (I , 4).

The BCA test predicts cytotoxic or necrotic effects of medical devices or materials with good c01Telation to animal experiments and high sensitivity (2, 3).

The test item is analysed for its leachable cytotoxic contents in the BCA test. Cytotoxic effects lead to a reduction of the proliferation rate of the cells. This leads to a reduction in the protein content of the cell culture as compared to the control cultures and is detected colourimetrically after a 68 – 72 h incubation period via the BCA test (5, 7).

The BCA reagents are comprised of the water soluble and stable BCA (Bicinchoninic acid) and an alkaline Cu^{2}+ solution. The amino acids cysteine, cystine, tryptophan and tyrosine, which are a constituent of every cell, bind to these reagents, i.e these amino acids reduce Cu^{2}+ to Cu+, which then binds to bicinchoninic acid to form a water soluble violet dye. The intensity of the dye correlates with the cell number in the culture.

This cell culture method is applicable for the cytotoxicity analysis of all medical devices and materials which are destined for implantation or come in contact with tissue or tissue fluids for a longer period.

Aim of the Study

This *in vitro* method analyses the cytotoxic potential of the test item. The test is carried out using the mouse cell line L929 cultured with different concentrations of an extract of the test item. The vitality of the cells or potential cytotoxic effects of the extract are registered via the protein content of the cell culture as compared to the controls.

Materials and Methods

Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor.

Name:	Surgical stainless steel tray coated with NanoMoldRelease coating
Reference:	Surgical stainless steel tray, uncoated
Batch No.:	not provided by the sponsor
Storage:	at room temperature
Expiry Date:	not provided by the sponsor
Safety precautions:	Routine hygienic procedures were sufficient to assure personnel health and safety

Preparation of the Test Item

Before extraction the test material and the reference were rinsed three times with sterile water and were rubbed down with a sterile tissue.

Extraction of the Test Item

The extraction was can-ied out in compliance with ISO 10993-5, -12. The test item was extracted for 22 h in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 ± 1°C. The surface/volume ratio in the assay was 6 cm^2/mL which c01Tesponds to 100% extract concentration. The extract was processed by sterile filtration.

Controls

Controls were set up in parallel to the test item cultures in order to confirm the validity of the test.

Negative control

The negative control, Polyethylene mate1ial (Art. No. 188.271, Lot 04080197, Greiner), was extracted at a weight/volume ratio of 1 g/5 mL medium for 22 h at 37 ± I °C.

Positive control

The positive control, Dimethylsulfoxide (DMSO 99.5%, Lot 6H007912, Applichem), was set up in a final concentration of 5% in DMEM 10% FCS.

Solvent control

A solvent control, consisting of the reference extracted in the extraction vehicle (DMEM 10% FCS) and treated in the same way as the treatment groups was included.

Cells

The test was cmTied out with L929 cells (ATCC No. CCLI, NCTC clone 929 (connective tissue mouse), clone of strain L (DSMZ)). The widely used cell line is known for its cloning efficiency and high proliferation rate.

For the test cells were cultured in 75 cm^2 culture flasks (Greiner) in DMEM (Biochrom) with 10% FCS-Gold (PAA) at 37 ± 1°C and 5.0% C02.

Dose Groups

I. Solvent control	Reference extracted in DMEM 10% FCS
2. Negative control	Polyethylene extracted in DMEM 10% FCS
3. Positive control	DMSO (5%) in DMEM 10% FCS
4. Test Item	6 concentrations of the test extract: 13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

Experimental Procedure

The extract of the test item and the solvent control were diluted five times with DMEM 10% FCS at a ratio of 2:3. 100 µL of the different dilutions or I00 µL of the controls were given to 3 parallel cultures in a 96 well plate (Greiner).

Log phase L929 cultures were washed and trypsinised with Trypsin EDTA for approximately 3 minutes. The enzymatic reaction was stopped with DMEM I0% FCS and a single cell suspension was made at a density of 1.0 x 10^5 cells per mL. 50 µL of this cell suspension were pipetted to all cultures with the exception of the blanks. The highest concentration of the extract in the cell cultures con-esponds to a surface/volume ratio of 6 cm^2/JnL. The cell culture plates were then incubated with the test extract for 69.25 h at 37 ± I°C, 5.0% $C0_2$ / 95% air.

BCA-staining

The protein contents of the individual cultures were measured colourimetrically using the BCA reagents (Uptima). The absorption at 550 nm was measured using a micro plate auto reader.

The mean absorption (Assonm) and standard deviation of the three parallel cultures was calculated and used for assessing the percentage of growth inhibition (% G.I.) following the depicted formula:

$$\% \text{ G.I.} = 100 - 100 \text{ x } \frac{(\text{Asso nm sample}) - (\text{Asso nm blank})}{(\text{Asso nm control}) - (\text{Asso nm blank})}$$

$A_{550\ mu}$ san1ple: **Absorption value of the test extract**
Asso nm blank: Absorption value of the blank cultures (without cells)
Asso $_{nm}$ control: **Absorption value of the solvent control**

Data Analysis

According to Borenfreund and Borrero (6) cytotoxic effects can be based on the protein content of the cultures, which is used as a measure for cell growth. Clear cytotoxicity is hereby defined as an effect leading to an inhibition of cell growth of more than 30% as compared to the cultures treated with solvent controls.

Deviations from the Project Protocol

Concerning: Name of the Test Item

Before:

Surgical stainless steel tray, coated

New:

Surgical stainless steel tray coated with NanoMoldRelease coating

Reason: Sponsor's request.

This deviation did not affect the quality or integrity of the study.

Results

	Rel. Pro1tein contmt (A550) (a)						Growth inhibition in %o
	1	2	3	x	±	s	
Blank	0.170	0.118	0.116	0.135	±	0.025	
Positive control (b)	0.232	0.222	0.223	0.226	±	0.005	87
Negative control (c)	0.972	0.943	0.899	0.938	±	0.030	0
Solvent control							
100% v/v	0.870	0.828	0.824	0.841	±	0.021	0
66.7% v/v	0.889	0.873	0.870	0.877	±	0.009	0
44.4% v/v	0.940	0.899	0.893	0.910	±	0.021	0
29.6% v/v	0.897	0.860	0.825	0.860	±	0.030	0
19.8% v/v	0.932	0.929	0.917	0.926	±	0.006	0
13.2% v/v	0.920	0.903	0.867	0.896	±	0.022	0
Test extract (d)							
100% v/v	0.784	0.820	0.719	0.774	±	0.042	9
66.7% v/v	0.846	0.843	0.837	0.842	±	0.004	5
44.4% v/v	0.896	0.912	0.869	0.892	±	0.018	2
29.6% v/v	0.858	0.819	0.850	0.842	±	0.017	2
19.8% v/v	0.923	0.871	0.870	0.888	±	0.025	5
13.2% v/v	0.868	0.894	0.869	0.877	±	0.012	3

(a) 3 para lidcultun s. mean ± standard deviation

(b) 5% DMSO in DM EM 10% FCS

(c) PE material extracted in D!VI EM !Oo/n FCS

(d) 11lc Lest item was {',X{1<1ct<:tl under agitation in DM Evt I 0% FCS lOr 22 h al 37 ± I " C and thc extract was culturcd !Or 69.25 h with L929 cells at a final surfacc/vo!u1ne ratio of 6 crn^2 test item / mL culture.

Discussion

Changes of cell proliferation due to the presence of cytotoxic substances were analysed in a cell growth inhibition test by comparing the protein content of the cell cultures treated with an extract of the test item with that of the untreated controls.

In the present study Surgical stainless steel tray coated with NanoMouldRelease coating was extracted under agitation for 22 h with DMEM 10% FCS. L929 cells were then incubated for 69.25 h with the following end concentrations of the extract:

13.2%, 19.8%, 29.6%, 44.4%, 66.7% and 100%.

The highest extract concentration corresponds to the IS010993-5, -12 described surface/volume ratio of 6 cm^2/mL.

Growth analysis of cells cultured with the test extract showed no relevant growth inhibition of L929 cells.

The controls confinned the validity of the study. Cell growth of the positive cultures was inhibited by 87%. The extract of the negative control did not show any inhibition of cell growth (0%).

Conclusions

In this study under the given conditions no leachable substances were released in cytotoxic concentrations from the test item.

Distribution of the Report

Sponsor	Ix (original)
Study Director	lx (copy)

References

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