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Research paper

Bio-Compatible Processing of LENS^{TM} DepositedCo-Cr-W alloy for Medical Applications

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Abstract

Developing a Medicinal implants or devices is a challenging task for the researchers, right from the selection of materials, design, bio-compatibility and implantation to the host tissue. At every stage it requires proper care in processing of medical implants. In recent years the demand for medical implants had grown rapidly due to the awareness in the society. Major share of implants is used by younger people as they are active in sports, motor vehicle accidents leads to facture. Even older people also preferring to implants for ease of living. The commonly used implants are, prosthetic joints, knee replacement, dental, maxillofacial reconstructions etc.There is huge demand for the medical implants in coming years, presently a few bio-materials available for implant devices such as Ti-alloys, Stainless steel and Co-Cr-Mo alloys. There a scope to the researchers to develop a new alloy that are bio-compatible in nature and bring down the cost of the implant procedure to the needed patients. In this context additive manufacturing (AM) is an advanced manufacturing technology emerging as prominent technique in medical fields. Laser Engineered Net Shaping^{TM} (LENS) is one such metal additive technique which provides fabrication of parts with the help of laser power, melts the powder alloy completely and builds parts layer by layer directly from the CAD model.

In the present study, samples are fabricated from LENS process and carried the *In-Vitro* and *In -Vivo* bio-compatible tests as cytotoxicity and sub chronic toxicity to verify the toxicants release and their sustainability as the medical implants by the LENS deposited Co-Cr-W alloy samples. From the studies it is observed that the alloy samples show acceptable result. MTT assay demonstrate that cell viability is better in Osteoblast cells compared to the Fibroblast cells. Osteoblast cells show slightly more viable to the cell treatment on the samples during the experimental period. Sub chronic toxicity conclude that LENS deposited Co-Cr-W alloy is not toxic in all the rats studied herein and did not produce any toxic signs or evident symptoms. LENS deposited Co-Cr-W alloy did not cause any lethality or produce any relative body organs weight and haematological studies didn't show adverse effects.

Key Words: *LENS^{TM}; bio-compatibility; cytotoxicity; sub chronic toxicity; medical implants; Co-Cr-W alloys.*

1. Introduction

Economic status of the people had increased since globalization; the quality of living life of people has changed, awareness on health consciousness had increased in developed and developing nations across the globe. Activities of peoples like sports, driving vehicles, defence etc. are resulting number of accidents. Increase in age of elderly people their potential to bear weight of body had decreased in bones, joints. Due to advancements in medical science increased since there is demand for medical implants to restore the damaged parts. Most commonly used medical implants in human body are maxillofacial reconstruction, cranial repairs, dental implants, bone fracture fixations, load bearing implants, prosthetic joints[1].

Medical implants should possess high mechanical strength, wear and corrosive resistance and bio-compatible to human body[2]. There are few materials available for biomedical applications. Materials such as Ti alloy, stainless steel, Ni based alloys; tantalum alloys and cobalt based alloys are commonly used for medical implants and medical devices. There is huge demand in medical industry for advanced materials for medical applications. An extensive research is to be carried out in the development of biomaterials for implants and devices. The use of Ni based alloys has been decreased due to its allergic reactions and may cause cancer tumours in some patients[3]. It is good to avoid Nickel in all medical usage. Medical grade materials should be bio-compatible and should not get any chemical reaction with the body fluids while inserted in to human body. They should exhibit good mechanical properties such corrosion resistance, wear resistance and mechanical strength [4]. Commonly used alloys in medical use are Ti alloy (Ti-6Al-4V)[5], stainless steel[6], tantalum alloys[7] which are commercially developed so for. There is scope for development of new alloys in medical applications. Recently Cobalt based alloys gaining much importance in development of medical implants such as orthopaedic, load bearing implants, knee joints, prosthetics and dental applications. Cobalt alloys are high resistance to corrosion, excellent mechanical properties and bio-compatible. Commercially available cobalt alloys are Co-Cr-Mo and Co-Cr-W alloys.

Fabrication of medical implants in conventional manufacturing process is complex to handle the multiple tasks like melting, tooling, milling etc. It is also difficult to prepare the moulds for

medical structures with complex profile. To address this limitation in conventional process rapid prototyping emerged as promising manufacturing technique to build the complex geometry directly with CAD system without much post processing as in conventional process[8].

Rapid prototyping / Manufacturing (RP/M) is a fabrication technique which builds 3D parts additively layer by layer from CAD data. This technology was developed in mid 1980s. RP processing methods are classified into three groups based on raw materials used to fabricate models from liquid resin, solid wires / sheets, metal alloy powder [9]. Some of the commercially popular RP technologies are Steriolithography (SLA) [10], Fused Deposit Modelling (FDM) [11], Selective Laser Sintering (SLS) [12], Laser Engineered Net Shaping (LENS^{TM}) [13].Recently laser has gained significant importance in fabrication of medical implant devices by solid freeform fabrication techniques such as Selective Laser Sintering (SLS) [12], Direct Laser Sintering (DLS) [14] and Laser Engineered Net Shaping (LENS^{TM}) [13] have been used for different metal alloys. These processing techniques shows convincing advantages over conventional manufacturing process in terms of ease of design, shape, size internal architecture of implant devices.

LENS^{TM} is a metal powder additive manufacturing technique was developed by Sandia National Laboratories, USA in mid-90's. A schematic representation of the LENS^{TM} process is shown in Fig.1. A high power Nd: YAG laser is used to melt the powder particles, are forced to deposit on the metal pool formed on the substrate, directly from computer aided design (CAD) models rapidly with minimal post processing[14-15]. Amit Bandyopadhyay et al.[17] have demonstrated the NiTi alloy medical implants by LENS^{TM} process. Félix A. España et al.[18] have carried out research on LENS^{TM} deposited Co-Cr-Mo alloy, Amit Bandyopadhyay et al.[19] Ti6Al4V samples fabricated on LENS^{TM}, all these studies suggest that LENS^{TM} processed samples for medical implant application can eliminate the stress shielding, poor interfacial bond between the host tissue and the implant, and wear induced bone loss, porous implants in load bearing implants to increase their *in vivo* life time .

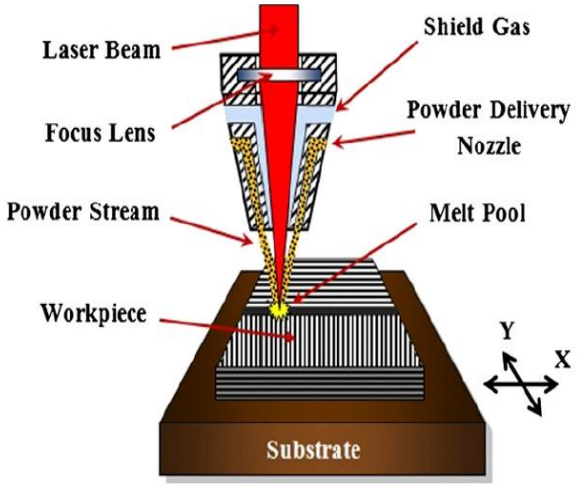

Fig. 1: Schematic representation of LENS^{TM}
Courtesy: Sandia National Laboratories

In this present work, samples are fabricated from Co-Cr-W alloys on Laser Engineered Net Shaping^{TM} machine. These samples are tested for *In-Vitro and In-Vivo* studies of cytotoxicity and sub chronic toxicity.

2. Materials and Method

2.1 Sample Preparation

Co-Cr-W alloy which is commercially available as Stellite – 6 (Kennametal Stellite GmbH Germany) in powder form with particle size 53 -150µm was used to fabricate samples. Laser Engineered Net Shaping^{TM} (MR-750, Optomec Inc., Albuquerque, NM, USA) is used to fabricate samples with dimensions 15x15x5mm as shown in Fig. 2. The process parameters selected for fabricating samples from the earlier studies of our work [19-20], with laser power 350W, scan speed 15 mm/sec and powder feed rate 7.5 g/min at these conditions samples are mechanical and electro chemical properties are accepted and shows best performing. The chemical composition of powder material in weight percentage is shown in Table. 1.

The laser deposited samples are then separated from the substrate by employing wire cut EDM (Ezeecut NXG). The samples are then polished on emery paper (silicon carbide) from grit size #220 to #1200 to attain smooth finish and scratch free surface.

Furthermore, these samples are polished to mirror finish on a fine cloth with diamond paste (9, 3 and1µm) as shown in Fig. 3.

Fig. 2: LENS deposited Co-Cr-W alloy samples

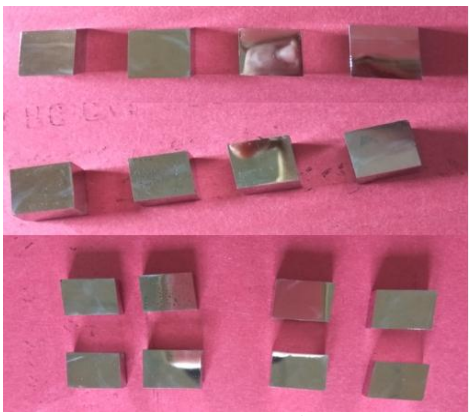

Fig.3: Post processed samples of LENS deposited Co-Cr-W alloy

Table 1: Stellite – 6 Chemical Composition in weight %

Elements	Kennametal	Chemical Analysis	
		Powder	Bulk
Cobalt (Co)	Base	Base	Base
Chromium (Cr)	28.0-32.0	30.8	31.2
Tungsten (W)	4.0-6.0	4.8	5.1
Carbon (C)	2.0-3.0	1.15	0.89

Silicon (Si)	1.2	0.4	1.3
Nickel (Ni)	1.0	2.7	2.2
Iron (Fe)	1.0	0.41	0.38

2.2 Cell Culture

Cell culture testing was conducted using BALB/c 3T3 mouse fibroblast cells grown in Dulbecco's Modifies Eagle's medium (DMEM) with 10% fetal bovine serum. Sufficient quantity of the cells will be cultured in bulk using the T75 cell culture flasks. If the cells are to be grown from cultures taken from liquid nitrogen storage, cryoprotectant will be removed. Cells will be sub cultured at least once before use. The cell line will be incubated at 37 ± 2°C in humidified 5% CO2 and 95.0 % air atmosphere. The cell line will be incubated to achieve approximately 80% confluency at 37 ± 2°C in humidified 5% (volume fraction) CO_2 and 95.0% air atmosphere as appropriate for the buffer system chosen for the culture medium for two to three days before the treatment [22].

2.3 Animals Preparation

For the *in vivo* analysis of bio-compatibility a rat model was developed and utilized. For this study animals were prepared according to ISO10993. All Sprague Dawley rats with age of 6-8 weeks were randomly assigned into two groups for consecutive 90 days corresponding to test material extract and the control vehicle. General anaesthesia was achieved using an intravenous injection. Each group is provided with 10 male and female rats respectively for the study. Body weights of the rats were taken with a week interval. All the experiments were approved by the Ethical Committee of the Bio-Logical Labs Pvt. Ltd, Hyderabad, India and were conducted following its guidelines.

2.4 Cytotoxicity (MTT- Assay)

The experiment will be performed by direct deposition of dilutions of test item extract on the sub confluent cell culture. After cell cultures are grown to a near-confluent mono layer in culture vessel, the medium aspirated and replaced with fresh complete media. The cell culture will be treated with 100%, 75% 50% and 25% of extract of each dilution of Test item. Cultures will be incubated for 24±2h to 72±2h at 37±1°C in humidified 5% CO_2 atmosphere. The treatment, positive, negative and vehicle controls will be done in triplicates. At the end of the treatment (24±2h to 72±2h incubation), spent media from (floater or detached cells will be discarded) and adherent culture will be trypsinized and collected in the respective labelled tube. Based on cell density the sufficient volume of cells suspension from each culture will be mixed with trypan blue (0.4-0.5%) and the number of viable cells (not stained) and nonviable cells (blue stained) are counted by using a haemocytometer.
Cell viability will be expressed as percentage of vehicle control number of cell excluding trypan blue. Determination of the cytotoxic effects will be done by qualitative and/or quantitative means as per ISO guideline (ISO-10993-5) biological evaluation of Medical implants/ Devices tests for *in vitro* Cytotoxicity.

2.5 Sub-Chronic Toxicity

The sub chronic toxicity evaluation was based on guidelines in the ISO 10993-11 for the biological evolution of medical implants/devices [23]. Forty Sprague Dawley Rats with equal number of both male and female were divided into two groups for vehicle control (10/sex) and the test material (10/sex). The rats were weighed and visual observations for mortality, behavioural pattern (Salivation, fur, lethargy, and sleep), changes in physical appearance, injury, pain and signs of illness were observed once daily during the experimentation period of ninety days. At the end of the experiment, all animals were anesthetized through intraperitoneal injection of a ketamine (60mg/kg).
The metal alloy samples with dimensions 5x2x2 mm are inserted to the animal by intraperitoneal injection, the rats were observed daily for their behaviour and body weight is noted weekly. Blood samples were collected from cardiac veins into nonheparinized and EDTA-containing tubes for biochemical and haematological analyses, respectively. The relative organ weight was calculated. Clinical pathology is performed haematological and biochemical analyses.

3. Results and Discussion

3.1 Cytotoxicity

MTT assay tests are used to determine the cell growth (proliferated) on the surface of the LENS deposited Co-Cr-W alloy samples. After running the assays on the ELISA plate reader (Synergy MX Bio-Tek, Instruments, Inc. USA), the data from the duplicate wells was averaged in order to obtain cells present on the surface of the alloy. Two types of cells are used as Osteoblasts, and human gingival fibroblasts to study the cell viability for 24±2h to 72±2h, for each day reading are noted and are Shown Fig. 4 in percentage of cell viability.
From the study it is observed that the Osteoblasts cell shows comparatives better cell viabilities than the fibroblasts cells. This might be the differences in oxide layers thickness and composition of the LENS deposited Co-Cr-W alloy samples[24]. The difference of cell viability between Osteoblasts and fibroblasts cells not much, variance of the two types of cell culturing factors as age (days) of media and possible disturbances to cell culture during the incubation.
Thus, cellular proliferation was supported equally well on the alloy samples. The results show better cell response by the Osteoblasts cells in those two cell cultures.

3.2 Sub-Chronic Toxicity

Sub chronic toxicity studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity and are designed to identify noob servable adverse effect level [25]. Sub chronic evaluation can also help to determine appropriate dose regimens for longer term studies. Consequently, in this study the sub chronic toxicity for LENS deposited Co-Cr-W alloy was evaluated in rats for 90 days.
Co-Cr-W alloy sample dose to the rat shows no adverse effect on the behavioural responses of the tested rats during the observation period. Physical observations indicated no signs of changes in the skin, fur, eyes mucous membrane, behaviour

patterns of the rats. There was no mortality observed at the tested dose nor was the weight loss in the rats affected. There are no significant differences observed in the relative organ weights compared to the control group in this study (Table. 2). The kidney function parameters did not reveal any relevant changes and liver function parameters were noted with the exception of marginal variations following administration of Co-Cr-W alloy samples.

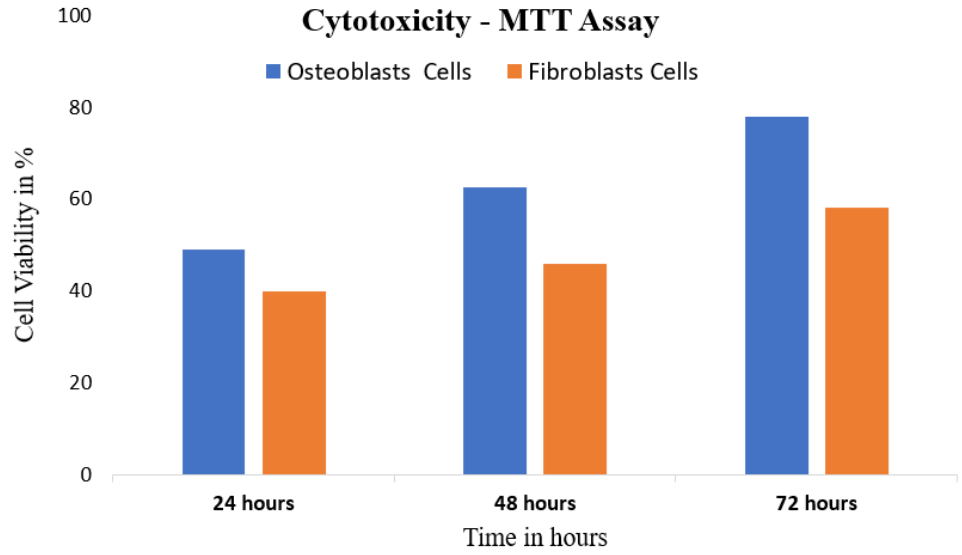

Fig. 4 Graphical representation of cell viability of Osteoblast and Fibroblast cells

From the present study it was seen that there was no significant change in the haematological parameters in the test sample treated group compared to the control vehicle group (Table. 3). Most haematology measures (haemoglobin, total red blood cells, red blood cells distribution width, total white blood cells, neutrophils, lymphocytes, monocytes and platelet count) in treated rats were not significantly different from the controls, with the exception of marginal variations in certain parameters.

Table 2: Relative organ weight (%) of animals

Body organs	Male Rat		Female Rat	
	Control	Treated	Control	Treated
Body weight (g)	494±36.3	492.3±54.2	291±41	288.35±33.1
Brain (%)	0.46±0.044	0.40±0.04	0.73±0.06	0.74±0.07
Pituitary gland (%)	0.002±0.001	0.003±0.001	0.005±0.001	0.006±0.001
Lung (%)	0.30±0.024	0.33±0.03	0.41±0.03	0.45±0.04
Heart (%)	0.28±0.023	0.25±0.02	0.33±0.02	0.32±0.02
Thymus (%)	0.061±0.016	0.09±0.02	0.09±0.02	0.12±0.03
Thyroids: left (%)	0.004±0.001	0.002±0.001	0.002±0.03	0.004±0.001
Thyroids Right (%)	0.002±0.001	0.003±0.001	0.003±0.001	0.004±0.001
Liver (%)	2.45±0.141	2.47±0.19	2.49±0.16	2.51±0.16
Kidney: left (%)	0.29±0.04	0.30±0.02	0.32±0.03	0.34±0.04
Kidney: Right (%)	0.29±0.03	0.30±0.03	0.32±0.02	0.34±0.03
Spleen (%)	0.124±0.018	0.15±0.01	0.17±0.02	0.18±0.02
Adrenal gland: left (%)	0.004±0.002	0.005±0.001	0.002±0.003	0.013±0.003
Adrenal gland: Right (%)	0.005±0.002	0.006±0.001	0.012±0.001	0.013±0.002
Testis/ Ovary: left (%)	0.034±0.08	0.311±0.051	0.014±0.005	0.012±0.007
Testis/ Ovary: Right (%)	0.034±0.08	0.310±0.074	0.013±0.004	0.013±0.005
Epididymis: left (%)	127±0.023	0.128±0.027	-	-
Epididymis: right (%)	0.135±0.03	0.136±0.038	-	-
Prostate gland (%)	0.14±0.02	0.12±0.02	-	-
Submaxillary gland (%)	0.13±0.01	0.14±0.02	0.17±0.03	0.18±0.02

Table 3: Haematological values of animals

Haematology	Male Rat		Female Rat	
	Control	Treated	Control	Treated
WBC (K/ul)	8.70±1.29	8.82±1.37	5.60±1.30	4.78±1.34
Neutrophils (K/ul)	2.02±0.58	2.05±0.48	0.73±0.25	0.78±0.29
Lymphocytes (K/ul)	6.25±1.77	6.23±1.76	3.9±1.26	3.88±1.25
Monocytes (K/ul)	0.31±0.11	0.29±0.12	0.19±0.10	0.17±0.08
Eosinophils (K/ul)	0.09±0.03	0.10±0.03	0.06±0.03	0.05±0.02
Basophils (K/ul)	0.01±0.02	0.01±0.01	0.01±0.001	0.01±0.01
Large unstained cells (K/ul)	0.02±0.02	0.03±0.01	0.03±0.02	0.02±0.02
RBC (M/ul)	8.68±0.36	8.72±0.51	7.68±0.36	7.84±0.19
Hemoglobin (g/dl)	15.2±0.44	16.4±0.4	14.3±0.5	15.6±0.9
Hematocrit (%)	47.6±1.8	45.2±1.6	42.8±2.2	43.1±2.4
MCV (fL)	51.3±1.62	53.1±1.9	53.9±1.66	55.3±2.2
MCH (pg)	34.9±0.1	35.8±0.2	36.5±0.4	36.1±0.6
MCHC (g/dl)	36.2±0.3	35.7±0.2	35.9±0.5	36.2±0.6
Reticulocyte (G/ul)	157.8±11.3	158.1±11.6	129.2±29.6	128.4±27.7
Platelet (K/ul)	1022±99.5	1024±110	963±85	959±83
PT (sec)	18.3±1.4	18.7±1.4	16.9±1.2	17.1±1.0
APTT (sec)	26.8±2.1	27.2±2.6	16.2±1.8	16.8±1.5

4. Conclusions

It has been shown that the LENS deposited Co-Cr-W alloy samples result in positive response to cellular activity during the experimental period of 24-72 hours. MTT assay demonstrate that cell availability is better in Osteoblast cells compared to the Fibroblast cells. Osteoblast cells show slightly more viable to the cell treatment on the samples. LENS deposited Co-Cr-W alloy shows good viability for connective tissues.

Sub chronic toxicity conclude that LENS deposited Co-Cr-W alloy is not toxic in all the rats studied herein and did not produce any toxic signs or evident symptoms at sub chronic toxicity studies. LENS deposited Co-Cr-W alloy did not cause any lethality or produce any relative body organs weight and haematological studies didn't show adverse effects. The preliminary results suggest promising alternatives for exploring the further studies required to test for bio-compatible.

The present study is preliminary studies to bio-compatibility testing, these preliminary results leads to further investigations where the ultimate goal is to commercialize the Co-Cr-W alloy for medical implants/ devices applications.

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