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INDIGENOUS UNCOATED AND HYDROXYAPATITE COATED COMMERCIALLY PURE TITANIUM FOILS FOR GUIDED BONE REGENERATION IN DEFECT SITES OF IMPLANTS – AN IN VITRO STUDY

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ABSTRACT

Use of barrier membranes for the regeneration of bone defects has significantly changed implant dentistry in the past years. The design of the membranes employed in this study plays an important role in establishing a healing environment by separating tissues during healing and thus providing space for regeneration of bone in cases of insufficient bone around implants, it acts like a tent in cases of extraction sites- which is a potential site for future implant placement thus reducing the problems of irregular ridge defect, and the rigidity of the material prevents tissue ward collapse of the membrane.

The present study was conducted to prepare the titanium in the form of foil, and tested for biocompatibility using fibroblast cells, hydroxyapatite coating was made on the foil, characterization by X-ray diffraction for both the uncoated and the coated foils was done, and osteoblasts adhesion test was done and viewed under scanning electron microscope both before and after the test.

Materials and methods: Commercially pure titanium foils were made using 16% hydrofluoric acid. 3×3 cm sheets were cut and dipped in the acid intermittently and alternatively dipped in water and wash the acid. For every minute of etching the material is measured using a micrometer to check the thickness of the foil. It is carried on until it is 60µ thick and uniform pores appear. Hydroxyapatite coating was done over the foil by dip coating in a solution made by a mixture of hydroxyapatite powder, water, glycerine, polyethylene glycol, and ethanol. The foil is dipped and withdrawn at 45◦ a speed of 10mm/ 15 seconds. Then the foil is dried in an oven at 100 ◦ and heat treated in a microwave oven for 45 minutes.

Results: The titanium foil was treated for biocompatibility using fibroblast cells, and the test showed that the material was biocompatible. After coating with hydroxyapatite, the uncoated and hydroxyapatite-coated titanium foils and the hydroxyapatite powder were characterized by x-ray diffraction and the dip coated foils clearly showed the hydroxyapatite peaks along with the substrate titanium peaks. Scanning electron micrographs of the uncoated foil revealed uniform distribution of through and through pores and scaly appearance in between pores. The coated foil revealed uniform distribution of hydroxyapatite coating. And the thickness of the hydroxyapatite in cross section was 50µ. Osteoblasts adhesion test was conducted on both the uncoated and the coated foils and scanning electron microscopic study was conducted. The micrographs revealed the adhered osteoblasts on the surface of both the uncoated and the coated test samples.

Conclusion: A new membrane material titanium foil was prepared by acid etching, and the material proved biocompatible in fibroblast culture study. The foil was dip coated with hydroxyapatite. X-ray diffraction showed foils with hydroxyapatite peaks along with the substrate titanium peaks. Scanning electron microscopic study revealed micro pores and scaly appearance of the uncoated foil, and for the coated foil it revealed a uniform coating of the hydroxyapatite. The surface roughness of the foil has given provision for the attachment of the

hydroxyapatite. Osteoblasts adhesion test was done and scanning electron microscopic study was conducted to view the adhered cells. The osteoblasts cells have adhered to the substrate of both the uncoated and the coated test samples. The results revealed that this study could give rise to a new generation of osseo conductive membranes for use in implant defect sites.

Key words: Titanium foils, Hydroxyapatite coated foils, Fibroblast culture, Osteoblast adhesion test, Scanning electron micrographs.

Introduction

"Everything has its time"

The dinosaurs had their time, and so will it once be said that amalgam had its time too.In dentistry, implantology currently is in "its time". Other than the discovery of osseointegration more than twenty years ago, the concept of guided bone regeneration represents the most important progress in implant dentistry.

This material helps in following cases:

1. In cases of insufficient bone around implants,
2. Acts like a tent over the graft material,
3. Preserves the clot formed by acting as a tent in cases of extraction site, which is a potential site for future implant placement.
4. The rigidity of the material prevents tissue ward collapse of the membrane.

The present study was conducted to obtain an improved uncoated and hydroxyapatite coated titanium foils, both of which can be an economic replacement for their costly counterparts.

Aim of the study:

1. To prepare the titanium in the form of foil.
2. To test biocompatibility of the material using fibroblast cells.
3. To make hydroxyapatite coating on the foil.
4. X-ray diffraction done to characterize the surface of both the uncoated and hydroxyapatite coated foils.
5. Scanning electron microscopic study done to evaluate the surface of both the uncoated and hydroxyapatite coated foils.
6. Osteoblasts adhesion test done to evaluate the effectiveness of the materials in osteoblasts adhesion which would potentiate the growth of bone around implants.
7. Scanning electron microscopic study following osteoblastic adhesion test.

Materials and methods:

1. Commercially pure titanium sheets- grade I (ASTM)
   (Midhani grade – Titan 12),
   99.8% - Titanium,
   0.2% - Iron,
   0.1% - Oxygen,
   0.05% - Nitrogen.
2. 16% Hydrofluoric acid
3. Glass beakers
4. Glass measuring jar
5. Pipettes
6. Micrometer
7. Acetone
8. Ethyl alcohol
9. Distilled water
10. Hydroxyapatite powder - 7.3%
11. Ethanol (99% pure)
12. Polyethylene glycol 600
13. Glycerol.

Equipments:

14. Ultrasonic cleaner,
15. Autoclave,
16. BPL micro convection system, BMC-900 T,
17. X-ray diffraction unit,

18. Scanning electron microscope, XL30 SEM, PHILIPS,

19. Splutter coater- Hitachi.

Methods:

1. **Preparation of titanium foil (fig: 1 a, 2a,2b,2c)**

The titanium sheets were cut to a dimension of 3cm×3cm. 16% hydrofluoric acid w/v was taken in a glass beaker, and the cut sheets are dipped in the acid intermittently, and it is alternatively dipped in water to wash the acid. For every minute of etching, the material is measured with a micrometer to check the thickness of the foil. This procedure is carried on until the material is 60μ thick and uniform pores appear.

2. **Ultrasonic cleaning of the foil:**

Foils are ultrasonically cleaned in

a) Acetone for 20 min,
b) 70% ethyl alcohol solution for 20 min and
c) Finally in distilled water for 20 min.

3. **Preparation of hydroxyapatite coating over the titanium foil (fig:1b, 2a, 2b, 2c):**

The ingredients of the dip-coating solution and their weight percentages are:

a) Hydroxyapatite – 7.3%
b) Ethanol – 66.2%
c) Polyethyleneglycol 600 – 2.2%
d) Glycerol 10.2%
e) Distilled water – 14.1%

Preparation of dip coating solution (flow chart no:1):

1. A mixture of hydroxylapatite and water is made and maintained for 8 minutes.
2. When this mixture is around 2 minutes, polyethylene glycol and ethanol are mixed in a separate beaker and maintained for 8 minutes.
3. Glycerol is added to the hydroxyapatite and water mixture and maintained for 2 minutes.
4. After this time the polyethylene glycol and ethanol mix is added to the mixture of hydroxyapatite, water and glycerol.
5. Then the whole solution is maintained for 4 minutes. The final solution is the dip-coating solution. Titanium foil is dipped and withdrawn at 45 degree angle in a freshly prepared dip-coating solution at a constant speed of 10mm/15 seconds. This procedure was repeated 2-3 times to increase the coating thickness.The coated foils were immediately dried in an oven for 45 minutes. The microwave oven used in this process is a BPL micro convection system.

4. **X-ray diffraction (fig 3a & 3b):**

The hydroxyapatite powder, titanium foil, and hydroxyapatite-coated foil were characterised by x-ray diffraction technique. The dip-coated titanium foils clearly shows the hydroxyapatite peaks along with the substrate titanium peaks.

Fibroblast culture

Materials and methods:

Materials :

1) Commercially pure titanium foils,
2) Distilled water,
3) Glaxo modification eagle's medium (GMEM)
4) Cell line: VERO cells.
5) Fetal calf serum were procured from HI media, India and stored at -20 degree Celsius.
6) Antibiotic stock: Antibiotic stock was prepared with the following antibiotics in distilled water and added to the medium in the following concentration.
   Gentamycin : 50 mg/lit
   Penicillin : 1,00,000 units/litre
   Streptomycin: 50-100 mg/litre
   Fungizone : 25μ / litre
7) Sodium bicarbonate stock: Sodium bicarbonate 8.8% solution (w/v) in phosphate buffered solution was autoclaved and stored at +4 degree Celsius until required.
8) Trypsin versene glucose:

Trypsin : 0.5 gm
Glucose : 0.2gm
EDTA : 10.2 gm
PBS : 200ml

Trypsin versene glucose was sterilized by filtration through 0.22 µ membrane filters, ale quoted and stored at -20 degree Celsius until required.

9) Phosphate buffered saline 10X
Sodium chloride : 80.0gm
Potassium chloride : 2.0 gm
Sodium hydrogen
Orthophosphate : 11.33 gm
Potassium di hydrogen
Orthophosphate : 2 gm
Distilled water : 1000 ml.

Phosphate buffered saline (10X) was prepared in sterile distilled water from this 1 litre of 1X was prepared and sterilized by autoclaving at 121 degree Celsius for 15 minutes at 15 lbs pressure.

Instruments:

1) Plastic culture plates:

6 well tissue culture plates (NUNC).

25 sq.cm and 75 sq.cm tissue culture bottles (Greiner labor technik) were prepared for propagation of the cells.

2) Glass wares: glass beakers,
Glass measuring jar,
Micro pippetes,
Test tubes,
All glasswares were of borosil.

Equipments:

1. Ultrasonic cleaner
2. Light microscope with camera attached
3. Incubator.

Procedure:

1. Sterilization of glassware's:

The glassware's were washed thoroughly, with mild detergents and rinsed thoroughly with distilled water, dried and covered with aluminium foil and sterilized in autoclave at 121 degree Celsius for 20 minutes at 15 lbs pressure or in hot air oven at 180 degree Celsius for 2 hours.

2. Preparation and sterilization of medium:

The powdered medium was dissolved in 960 ml of distilled water, tryptose phosphate broth (10%) was added and the pH was adjusted to 7.4 with sodium bicarbonate and 2.3 ml/L of antibiotic stock was added and sterilized by filtration through 0.22µ membrane filter and stored at+4 degree Celsius.

3. Maintenance of VERO cells:

VERO cells were maintained in growth medium (GMEM 8% FCS and antibiotics). VERO cells were passaged at a split ratio of 1:3 on every 3-day. Briefly spent medium from bottles (25 sp cm), containing confluent monolayer were decanted and the cell layer washed with trypsin versene solution twice. Detached cells were harvested in 3ml of growth medium and resuspended to make 24 ml of cell suspension in growth medium. Cell suspension was seeded into 3 bottles (25-sq cm) at the rate of 8 ml per bottle and incubated at 37 degree Celsius.

4. Extraction:

The extraction liquid resulted from the incubation at 37 degree Celsius for 120 hours of the material in the extraction "vehicle" (minimum essential medium) under specific conditions.

A blank extraction was done using Glaxo modification earl's medium under the same conditions, except for the absence of material.

The cells were tested by direct contact of extraction liquid of the test implant material.

5. VERO Cyto toxicity study:

This was performed in 6 well tissue culture plates (NUNC). Cells were seeded at the rate of 2×10^5 cell/ml. 1.5ml of trypsinised cells were added with equal volume of each of the four metal extract in growth medium. 1.5ml of this

mixture was seeded into one well of the plate. Four such extracts and negative controls were tested in a single plate. Cytopathic effect was observed daily for four days. At the end of which the cells were stained with Giemsa.

Result of the test (fig 4a & 4b):

VERO cyto toxicity: none of the metallic extracts caused any cytopathic changes in the VERO cells, when the extracts were used as neat.

Osteoblasts adhesion test

Materials:

1. Disodium phosphate,
2. Sodium dihydrogen phosphate,
3. Phosphate buffer (0.1 M, pH=7),
4. Glutaraldehyde : Make up 2.5 ml 25% glutaraldehyde to 25 ml using phosphate buffer,
5. Isopropyl alcohol ,
6. Isoamyl acetate,
7. Trypsin,

Instruments:

1. Multiwell plates,
2. Glass beakers,
3. Pipettes,
4. Glass measuring jar,

Equipments:

1. Laminar flow bench,
2. Carbon di-oxide incubator,
3. Inverted phase contrast microscope,
4. Scanning electron microscope.

Procedure:

Cell culture system:

Osteoblasts were maintained with minimum essential media supplemented with foetal calf serum.

Maintenance of cells:

1. When the cells become confluent, subculture the cells using trypsin.
2. Incubate until the cells become rounded and begin to detach by checking under microscope every one minute.
3. Add 4ml of serum containing medium and disperse the cells carefully using a Pasteur pipette.

Adhesion test:

1. Test samples were conditioned with media.

2. Cells were seeded on to the conditioned test samples, standardized to 1.5 x 1.5cm, in multiwell plates with required amount media.

3. Incubate the culture for 24 hours.

4. After 24 hours, discard the medium from the culture dish.

5. Rinse the cells with phosphate buffered saline.

6. Fix in 2.5% glutaraldehyde solution in phosphate buffer for three times.

7. Dehydrate in different grades of alcohol.

8. Immerse in asoamyl acetate for 2 minutes.

9. Then processing is done for critical point drying, gold coating and observation under scanning electron microscopy.

Result of the test (fig: 5a,5b,5c, 6a, 6b, 6c):

Osteoblasts adhered on to both coated and uncoated test samples. Cell covered surface is seen in scanning electron micrographs of 400x. At higher magnification spreading of an individual cell can be seen.

RESULTS

The result in the present study indicates that a possible new way of thinking about implant placement may be on the horizon.

The foil prepared was 60µ thick with uniform distribution of pores, which can act as a passage for the nutrients to reach the underlying tissues.

Four samples of 15mm X 15mm dimension were subjected to fibroblast culture study. Extract of the material was prepared and tested for cytotoxicity. None of the metallic extracts caused any cytopathic changes in VERO cells

when observed for four days. Thus proving the biocompatibility of the material.

The hydroxyapatite coating was done by preparing a dip coating solution. When assessed by X-ray diffraction the coating material was identified as hydroxyapatite and the substrate as titanium.

Scanning electron microscopic study revealed that the uncoated material had distribution of pores that were through and through, the area in between the pores appeared scaly and rough, proving the ability of the material to retain the hydroxyapatite coating. In the coated material, hydroxyapatite was uniformly distributed on the substrate. The vertical section showed that the thickness of the hydroxyapatite material was 50µ.

Osteoblast adhesion test was performed for both coated and uncoated test samples. 1.5 x 1.5 cm test samples were conditioned with media and cells were seeded on the samples in multiwell plates. Step wise procedures were conducted and observed under scanning electron microscope.

Micrographs showed that the osteoblasts had adhered onto both the coated and the uncoated test samples. Cell covered surface was seen, and at higher magnification and spreading of an individual cell was seen.

DISCUSSION

The term guided bone regeneration, has been used in tissue engineering for some years and is actually a specialized sub-area of tissue engineering and using this procedure for treatment of partial and total edentulism with dental implants has become an accepted treatment modality.

Guided bone regeneration is based on the concept that most tissues are capable of self-reconstitution if appropriate conditions exist and by compartmentalizing wound healing. By placing a physical barrier between epithelial and connective tissues on one side and implants and bone on the other side, guided bone regeneration procedures aim to create a protected space for the blood clot to form and organise. The presence of a cell occlusive membrane is required to prevent the migration of epithelial and connective tissue cells into the wound area, thus allowing bone cells to form from marrow spaces to repopulate the defect and to mature into new bone.

Membranes used in guided bone regeneration should possibly achieve a good degree of tissue integration with neighbouring connective tissues in order to obtain a mechanically stable environment necessary for successful bone and soft tissue healing.

However regeneration of the bone tissue may be influenced by the nature of the membranes themselves and regeneration takes place in sequence of events including blood clot formation, angiogenesis, osteoprogenitor cell migration, woven bone formation, compaction of woven bone and secondary remodelling.

In this study, we have made indigenous commercially pure titanium foils by etching, until it is 60µ thick and uniform pores are seen. Hydrofluoric acid is used because it attacks titanium even at very dilute concentrations.

Hydroxyapatite coating is done on the foil by dip-coating, in a solution prepared by a combination of hydroxyapatite powder, ethanol, polyethyleneglycol, glycerol, and distilled water. Hydroxyapatite is used because of its osteoconductive and osteophilic properties.

Assessment of fibroblast culture: The titanium was tested for biocompatibility using fibroblast cells. Metal extract was prepared by incubation at 37° C for 120 hours of the material in the extraction vehicle. Trypsinised VERO cells were added with equal volume of

the metal extract in the growth medium. 1.5 ml of this mixture is added to 5 wells of the six well plate and negative control were tested. The cells were observed daily for four days. None of the metal extract caused any cytopathic changes, thus proving the biocompatibility of the material.

Assessment of x-ray diffraction: The hydroxyapatite powder, titanium foil and hydroxyapatite-coated foil were characterized by x-ray diffraction. The uncoated foil clearly shows the titanium peaks and the coated foil shows the hydroxyapatite peaks along with the substrate titanium peaks.

Assessment of osteoblasts adhesion test: Although the study of fibroblasts provides valuable information about its response to exogenous materials, cells of different origin react differently to foreign bodies. Thus, the results from fibroblasts should not be transferred to bone cells.

The uncoated and the coated samples were tested for osteoblasts adhesion. Cells were seeded on to the surface of the materials. The material along with the medium was incubated for 24 hours. Later the medium was discarded from the culture dish and processed for critical point drying, gold coating and observed under scanning electron microscope.

The surface chemistry of biomaterials can directly affect cell behaviour and interactions between the host environment and the biomaterials may have significant downstream consequences. Therefore the nature of the materials used in implantology may drastically influence surrounding bone regeneration.

Cell attachment and migration are dependent on cell/ substrate interaction. The surface properties of the material have a strong biophysical influence on the cell kinetics. Osteoblasts are an anchorage – dependent cell type and need to attach and spread so as to divide and become confluent.

The cytoskeletal response (actin filament and focal contact formation) of osteoblast-like cells to various substrates has been studied by many investigators.

Bone cells are known to secrete a great amount of transforming growth factor-β (TGF - β_1) and express cell surface TGF – β_1 binding sites.

Produced at all stages of bone remodelling, TGF – β_1 is a potent mitogen in osteoblast-enriched cultures from foetal tissue. It increases the expression of many genes associated with osteoblast activity and the production of extra cellular matrix macromolecules such as type I collagen, fibronectin and osteonectin.

Hasegawa and coworkers[1] demonstrated that the conformational state of proteins can influence cell activity and consequently extra cellular matrix formation. This might be one of the reasons why cells, because of more favourable spreading reaction, increased their rate of growth and supported the formation of mineralized matrix.

The role of TGF-β_1 in bone regeneration is mediated by changes in extra cellular matrix macromolecules, which, in turn, are regulated by a balanced secretion of biologically active growth factors.

Human osteoblast synthesis of these growth factors and extra cellular matrix macromolecules may be influenced by the materials used in barrier membranes.

Guided bone regeneration is a means of using the osteogenic potential of progenitor bone cells, to create new growth in a variety of osseous defects. Osteogenesis on bioactive substrates in characterized by a temporal sequence of biologic events involving cell morphology, proliferation and differentiation.

Hurzeler and associates[2] found a substantial amount of "re-osseointegration" after treating ligature peri-implantitis using a combination of guided bone regeneration and bone grafts.

Assessment of scanning electron micrographs before osteoblasts adhesion:

The coated samples exhibited a thickness of 50μm of hydroxyapatite coating in cross section. When the surface was viewed, the hydroxyapatite crystals were uniformly distributed and uniform in size. The surface of uncoated foil revealed uniform distribution of pores and the surface in between the pores had a scaly appearance, which aided in both adhesion of osteoblasts and hydroxyapatite coating.

Assessment of scanning electron micrographs after osteoblasts adhesion: The micrographs revealed that osteoblasts adhered to both the uncoated and the hydroxyapatite coated samples. Cell covered surfaces were seen in the micrographs of 400x. at higher magnification spreading of an individual cell was seen.

Thus the material used in this study has proved to be osseoconductive by promoting the adhesion of osteoblasts to the substrate.

The present study is an in vitro study. For further clinical application an in-depth invivo study is of paramount importance.

Summary

The study was conducted to prepare a titanium foil, which can be used as a membrane for guided bone regeneration in defect sites of implants.

Titanium sheets of required dimensions were cut and immersed in hydrofluoric acid intermittently and measured every minute of etching using micrometer until the foil is 60μ thick and distribution of micropores is present.

The material was subjected to fibroblast culture to evaluate the biocompatibility. The results indicated that the material is biocompatible.

The prepared foils are dip-coated, and the uncoated and the coated foils were characterized by X-ray diffraction technique. The uncoated foils clearly shows the titanium peaks and the dip-coated titanium foils showed the hydroxyapatite peaks along with the substrate titanium peaks.

Scanning electron microscopic study showed micropores in the uncoated material and the scaly substrate in between the pores. And the hydroxyapatite coated material showed uniform coating of the material.

Osteoblasts adhesion test was conducted on both the uncoated and coated test samples. The scanning electron micrographs revealed the adhered osteoblasts on the surface of both the test samples.

CONCLUSION

1. A new membrane material, titanium foil was prepared.

2. The material was proved biocompatible in fibroblast culture study.

3. This material was dip-coated with hydroxyapatite.

4. X-ray diffraction showed foils with hydroxyapatite peaks along with hydroxyapatite peaks along with the substrate titanium peaks.

5. Scanning electron microscopic study revealed micropores and scaly appearance of the uncoated foil, and uniform coating of hydroxyapatite.

6. The surface roughness and pores has given provision for the attachment of the hydroxyapatite crystals.

7. Osteoblasts adhesion test was done and scanning electron microscopic study was conducted to view the adhered cells.

8. The osteoblast cells have adhered to the substrate of both the coated and uncoated foils.

9. At higher magnification spreading of an individual cell was seen.

The results reveal that this study could give rise to a new generation of osseoconductive membranes which can be used in various clinical conditions.

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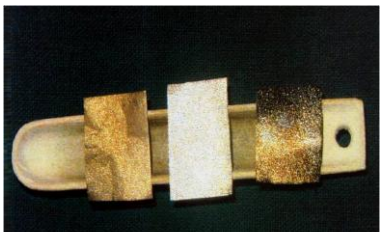
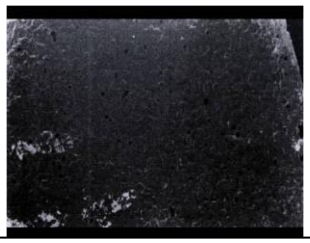
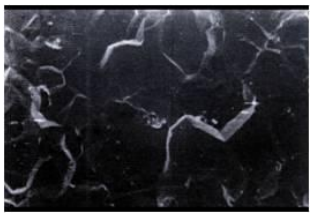
LEGENDS

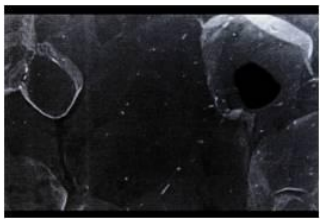


1a. Uncoated commercially pure titanium foil

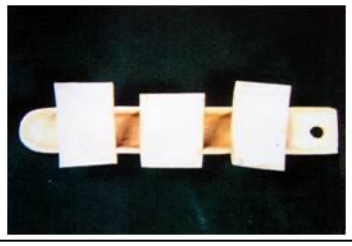


1b. SEM Study - Uniform distribution of pores at 20 magnification

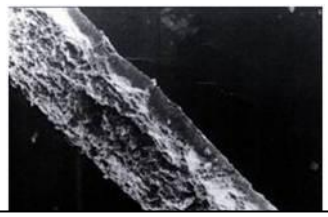




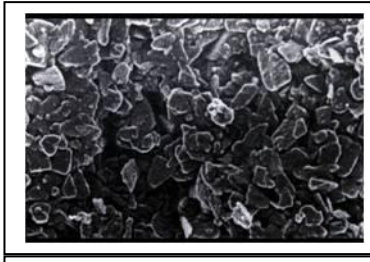
1c & d
At 300 and 500 magnification respectively scaly appearance of material between the pores reveals a rough surface.

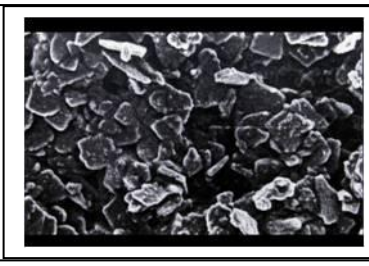


2a. Hydroxyapatite coated titanium foil

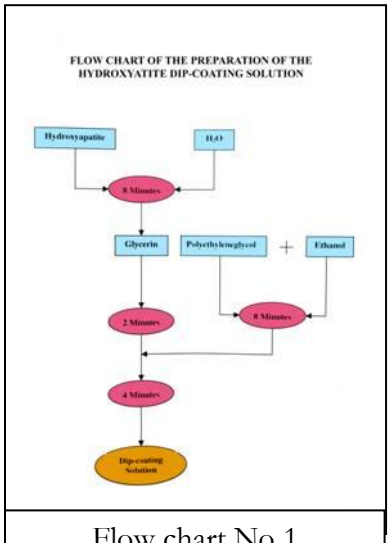


2b. SEM study -In cross section 50µ thickness of hydroxyapatite coating is seen

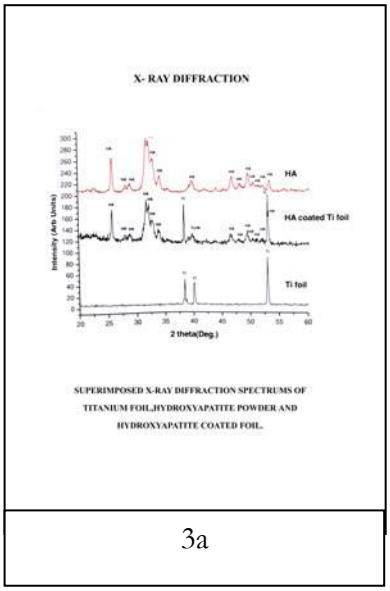




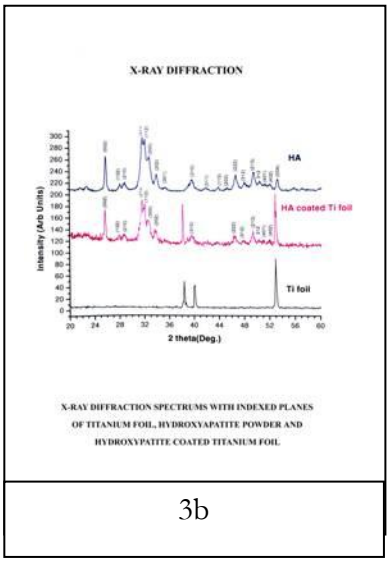
2c & d
At 300 and 500 magnification respectively uniform distribution of hydroxyapatite crystals are seen



Flow chart No 1



3a

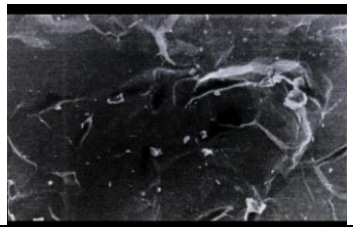


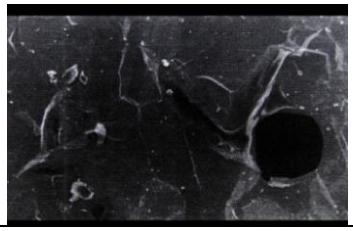
3b

4a . Cell rounding during trypsinization

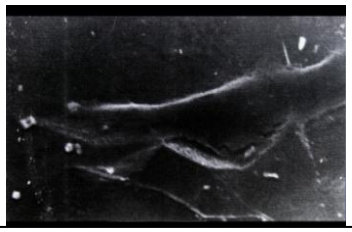
4b. Cells were well rounded and no sign of cytopathic changes

Scanning electron micrographs after osteoblast adhesion test – uncoated foils



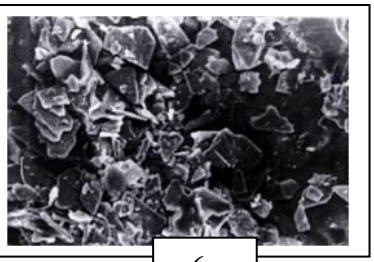


5a .& 5b.
At 400 magnification cell covered surfaces is seen

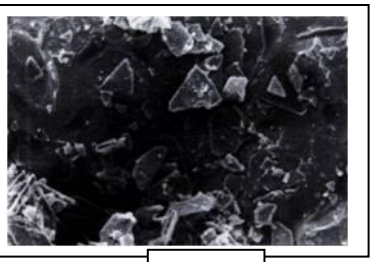


5c. At higher magnification spreading of an individual cell is seen

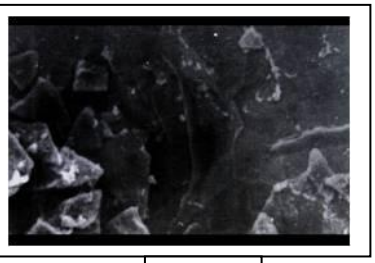
Scanning electron micrographs after osteoblast adhesion test – hydroxyapatite coated samples



6a



6b



6c