Proangiogenic Nano-Biomaterials for Bone Tissue Engineering

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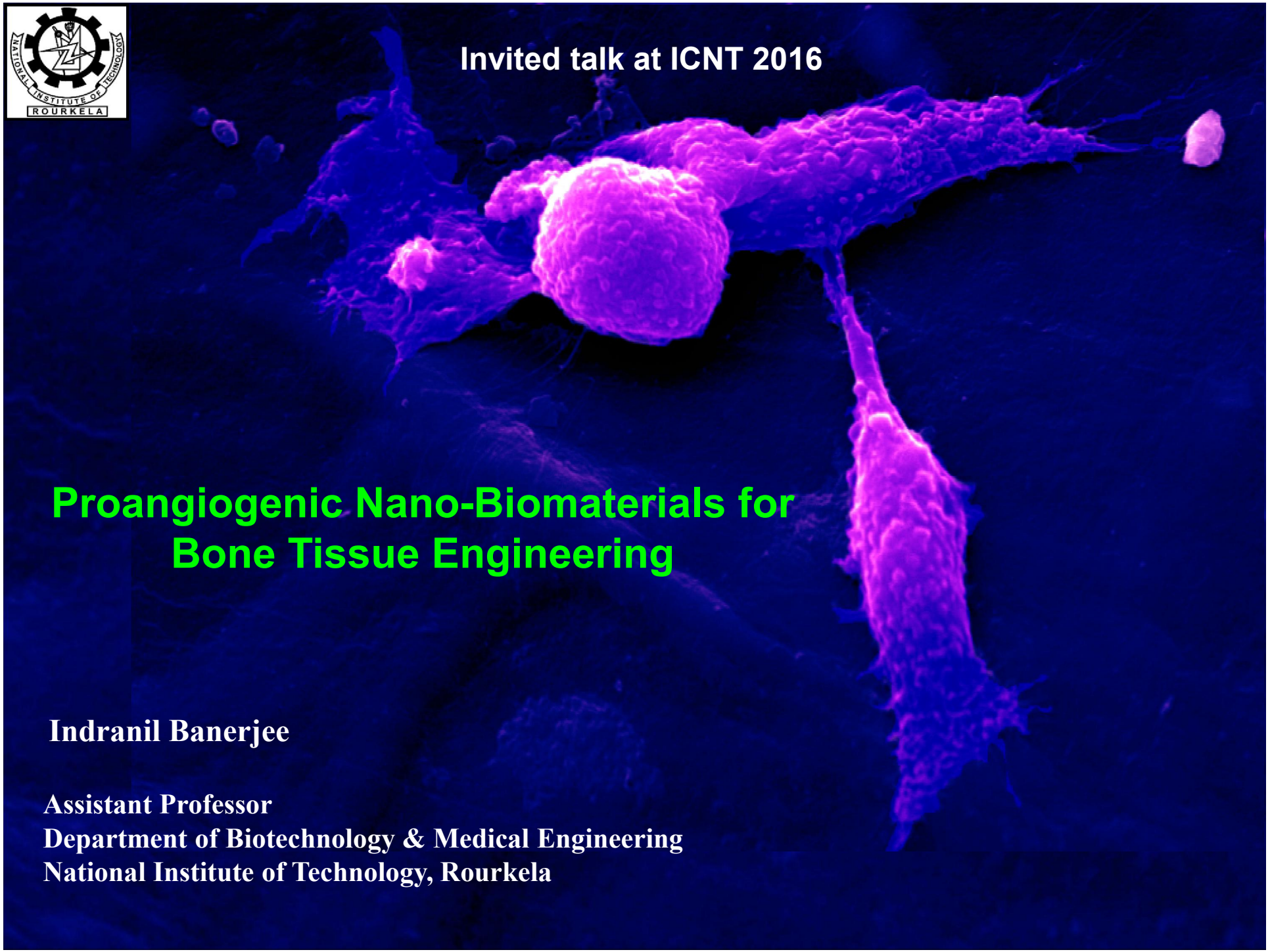
Effective replacement / restoration of traumatized, damaged or lost bone is a major clinical and socio-economic challenge. In recent years, emergence of the bone tissue engineering (BTE) as a therapeutic alternative of conventional clinical modalities especially as a replacement of auto- and allo- grafting has brought new hope in clinical orthopaedics. The success of the BTE depends on the integration of the biologically active osteogenic scaffolds (with / without bone cells) to the native osteo-chondral system after implantation at the injury or defect site. It has now been confirmed that such integration process seemingly relies on angiogenesis, which leads to the generation of vascular network in the neo-bone tissue. Angiogenesis is essential for the delivery of nutrients and gases to the cells present at the distal location of an implant, which can hardly be reached through interstitial fluid diffusion. Plenty of clinical evidence showed that impaired vascularisation results in atrophic non-union of the bone. Keeping this perspective in mind, people have adopted diverse strategies to improve the extent and the quality of angiogenesis in BTE. However, those strategies have failed to be a commercial success because of number of factors including cost, technical difficulties, genotypic variation of cells and potential health risks. To overcome the stalemate, research focus has now been shifted towards the angiogenic biomaterials, materials that can stimulate the cells for biased production of angiogenic factors, both *in vitro* and *in vivo*. Different research groups has now working on the development of angiogenic biomaterials. We are one of the leading research groups, involved in developing low cost proangiogenic nano-hydroxyapatite (nHAp). Hydroxyapatite is the most common bioceramic used in bone tissue engineering because of its chemical resemblance with bone apatite. We have adopted a novel strategy that leads to the angiogenesis through the activation of tissue hypoxia mimicking HIF-1α pathway. Following that strategy we have developed several types of proangiogenic hydroxyapatite either by doping 'group - d' bivalent ions like Co^{+2} , Ni^{+2} in nHAp crystal or by conjugating the hydroxyapatite with natural biopolymers like gum tragacanth. All the chemically modified nHAp are subjected for extensive physico-chemical characterization that includes XRD, FT-IR, SEM, TEM, BET, DLS and Zeta potential analysis. we confirm the osteo-conductive property of the modified nHAp by checking the response of the osteoblast cells (MG-63) in vitro. For this purpose , detailed studies pertaining to the cell viability and proliferation (MTT and flow cytometry based live -dead assay, cell cycle analysis), and cell differentiation (done by RT-PCR and Western blot) was done. We also test the osteogenic properties in vitro using human mesenchymal stem cell. The angiogenic property especially the expression of cellular VEGF and its related mechanistic pathways is proved in both MG-63 cell line and in human mesenchymal stem cells. We finally confirm the formation of endothelial linkage in vitro through tube formation assay. We believe that these set of experimental evidences will help the researcher in designing and developing proangiogenic biomaterials in coming future.



Invited talk at ICNT 2016

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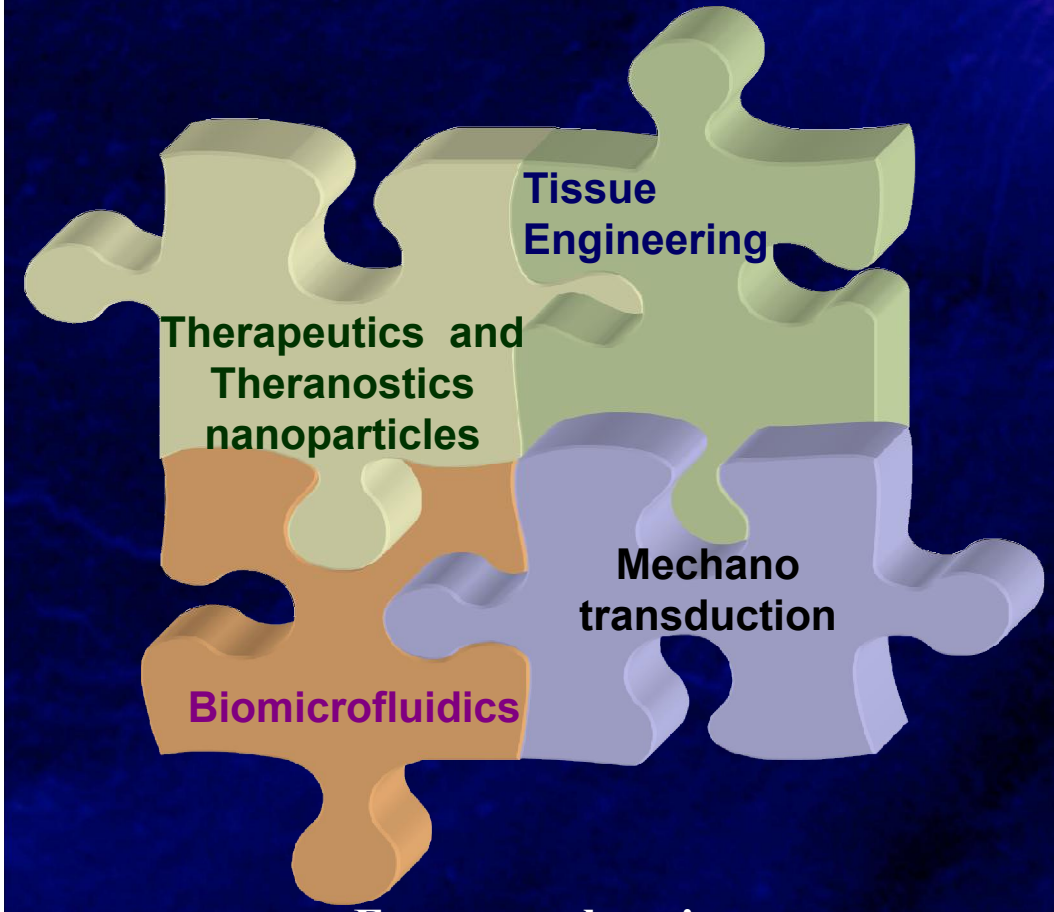
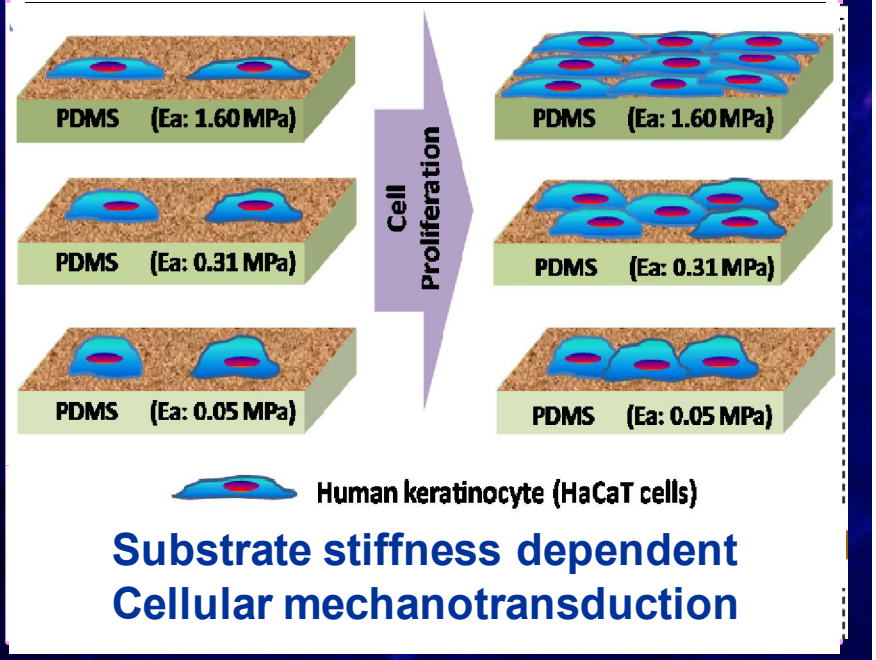
Research interest and area of expertise



Research Activity



Substrate stiffness dependent Cellular mechanotransduction

Extramural projects:

1. **Microfluidics based interstitial flow-mimetics. (Funded by DBT)**
2. **Group project under Center of Excellence in Orthopedic Tissue Engineering and Rehabilitation . (Funded by MHRD)**
3. **Cell based suspension array on magnetic-upconversion barcodes. (Funded by DBT)**

Ves Flow

Approaches to induce angiogenesis and its limitations

- **Application of angiogenic growth factors like VEGF and PDGF**
  - **Limitation :**
    - **Short half-life**
    - **high cost**
    - **uncontrolled 3D distribution**

- **Cell based approach**
  - **Limitation :**
    - **Phenotypic variation exists among the endothelial cells depending on source, organ and tissue location**
    - **Controlled co-culture of a number cells with endothelial cells**

- **Micro patterning and microfluidics**
  - **Limitation :**
    - **Sophisticated and costly infrastructure**

- **Gene therapy**
  - **Limitation :**
    - **Targeting specific host cells**
    - **introduction of vector gene into the patient**

Quest for an alternative…..

Our proposition

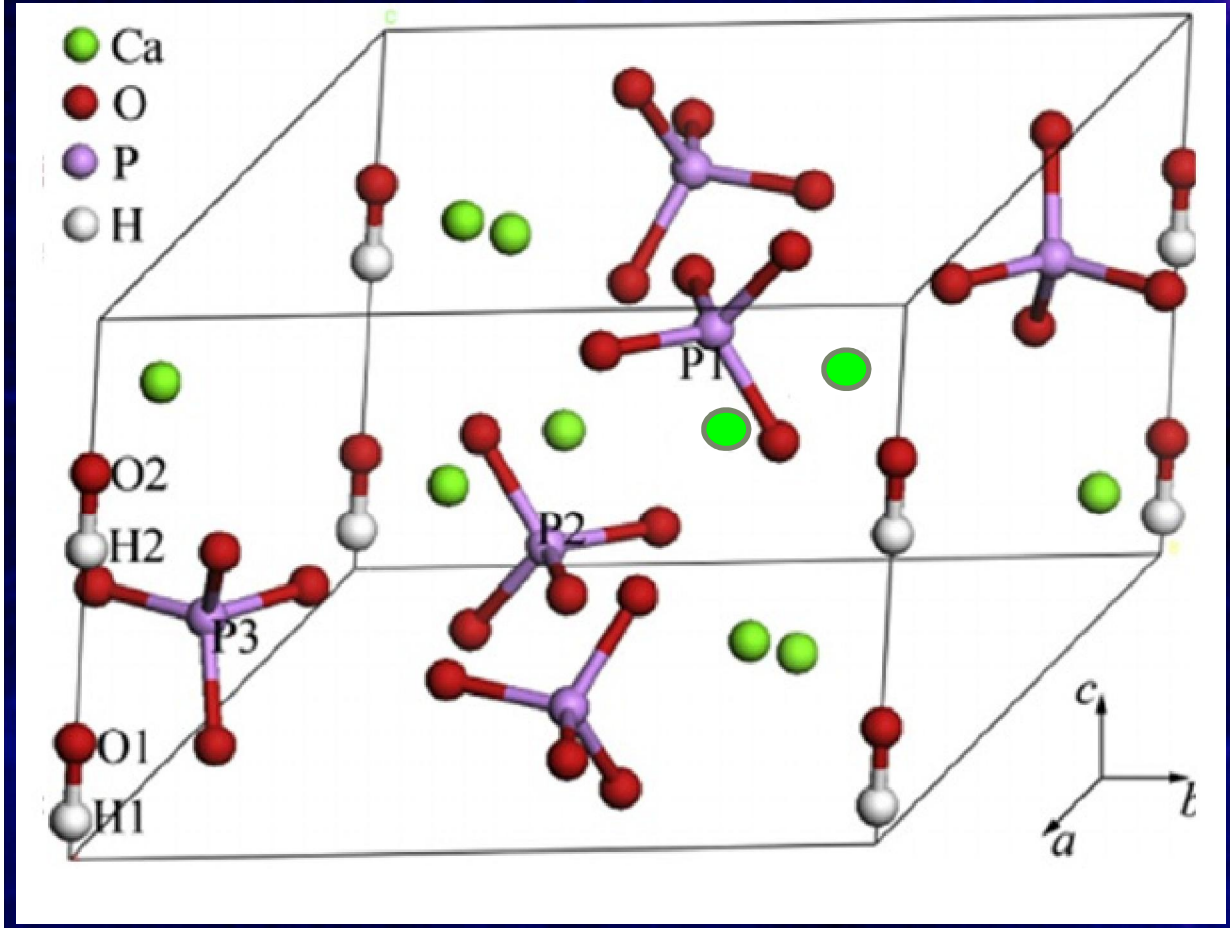
Proangiogenic Biomaterial

A biomaterial capable of stimulating the cells to express angiogenic factors.

The big question is

How to get it or how to make it ???

Synthetic nano hydroxyapatite : Our template material



- Biocompatible
- Non immunogenic
- Osteo-conductive
- Chemical and structural resemblance with bone apatite

M^{+2}

Out line of the work ….

Development of proangiogenic nano hydroxyapatite by

(i) Doping of Ni^{+2} ions

(ii) Conjugating gum tragacanth

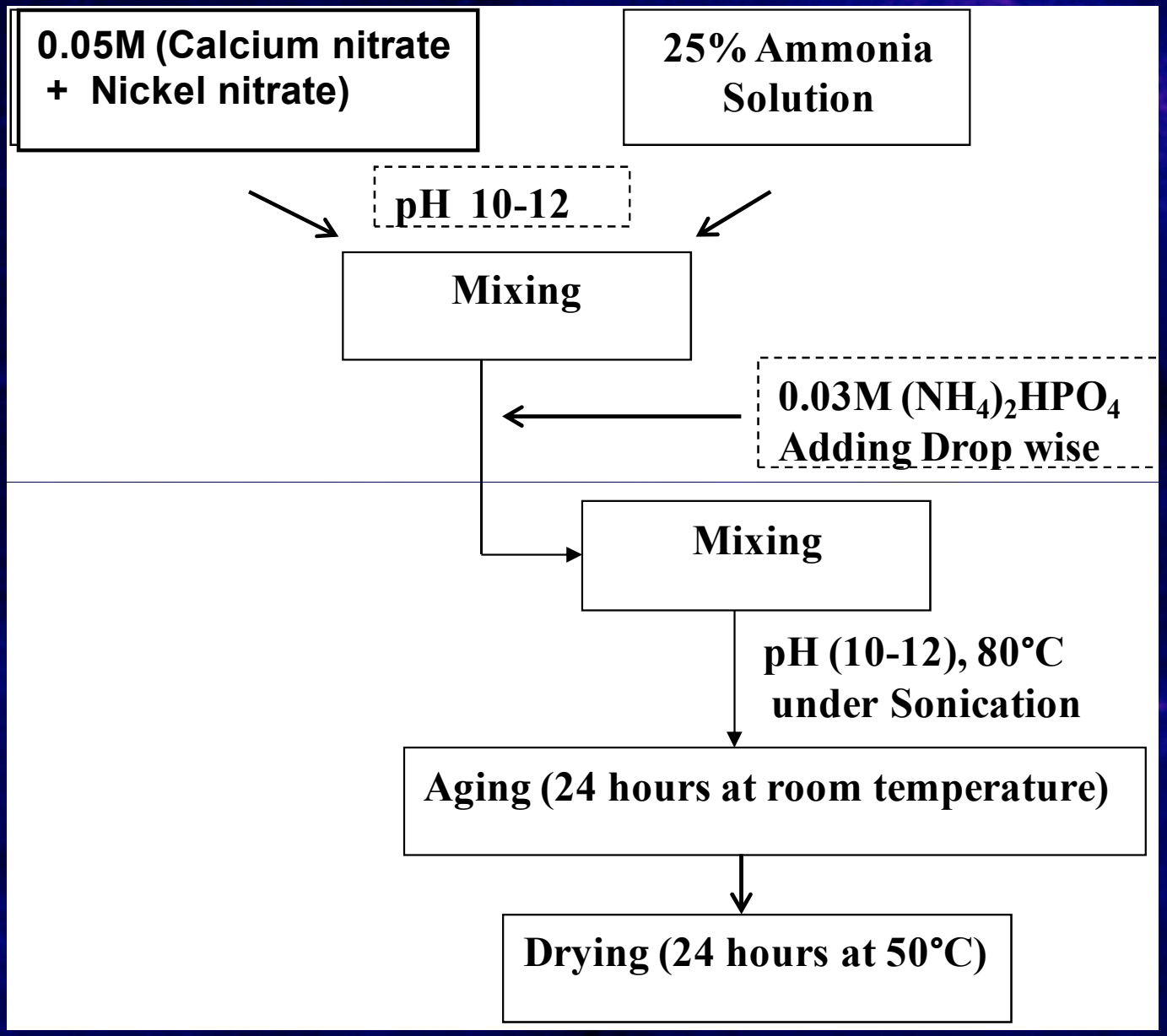
Synthesis

Physico-chemical characterization

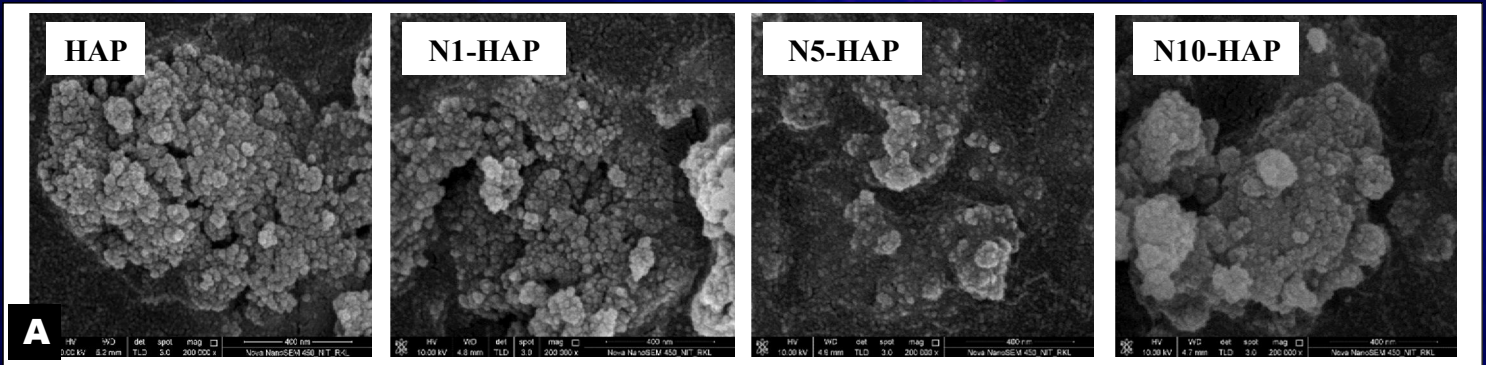
Study of osteo-conductivity

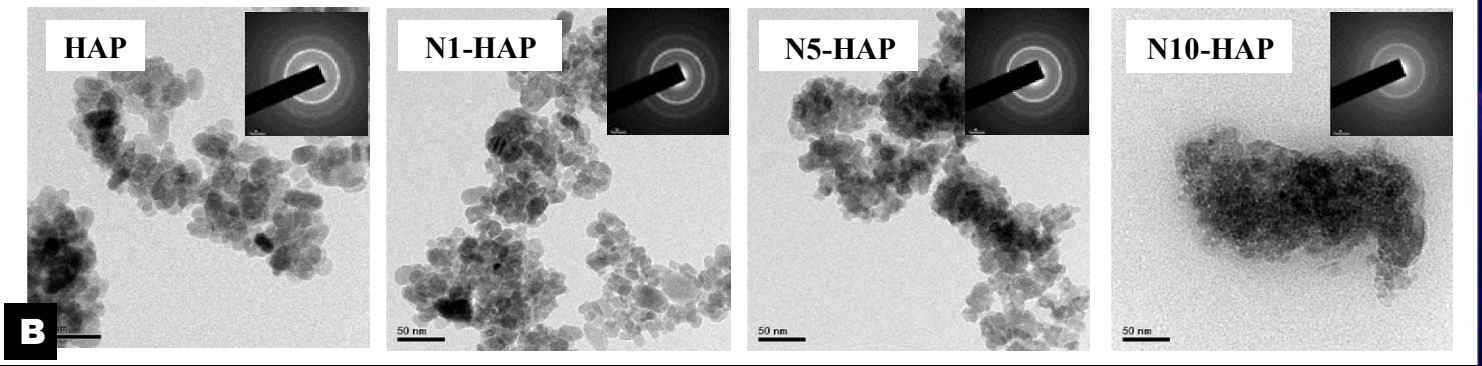
Study of angiogenic property

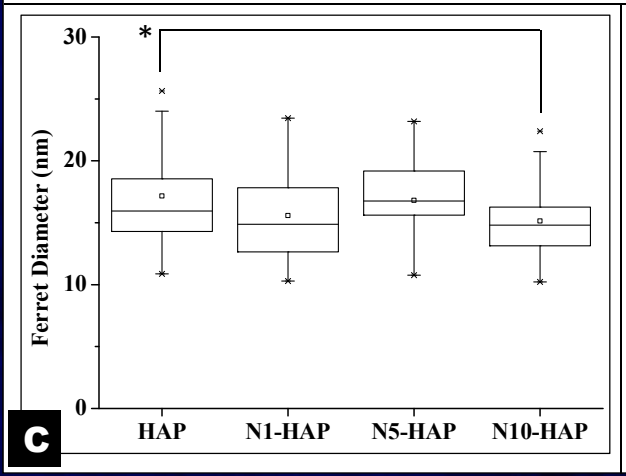
Synthesis of Ni^{+2} doped nano hydroxyapatite



Analysis of morphology, size and extent of doping







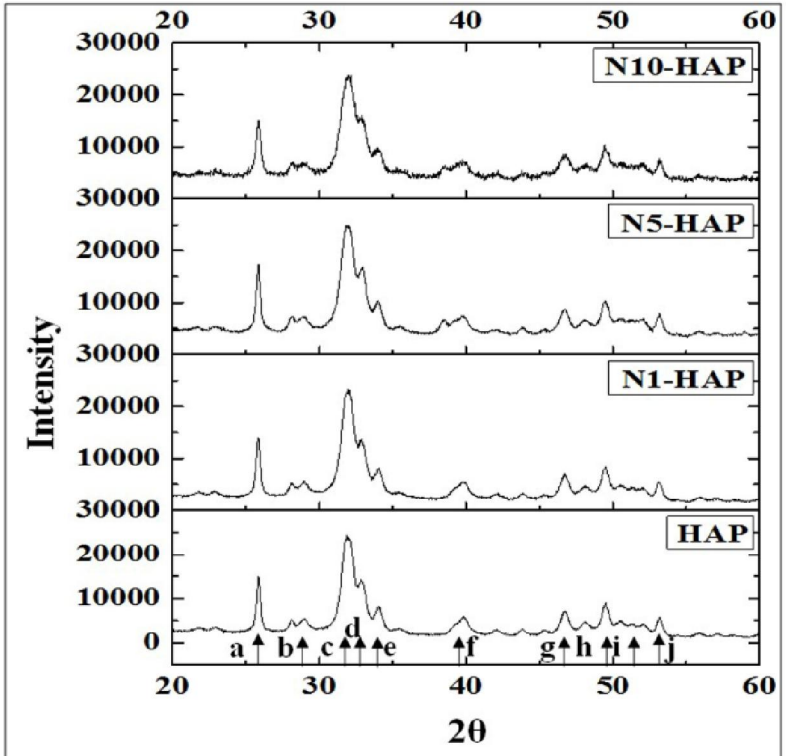
ICP-OES Analysis: Nickel doping in HAP samples

Sl.No	Samples	Doping (Theoretical) (w/w %)	Doping (Actual) (w/w %)
1.	HAP	-	-
2.	N1-HAP	1	0.26 ± 0.006
3.	N5-HAP	5	0.87 ± 0.019
4.	N10-HAP	10	2.97 ± 0.21

D

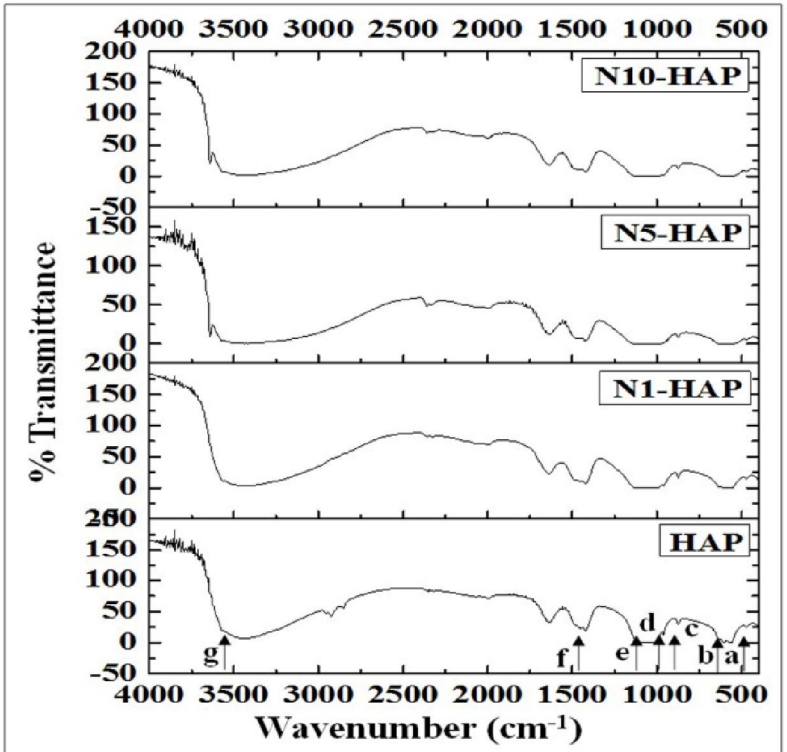
Micrographs of nHAp and Ni $^{+2}$ doped nHAp [A] FESEM , [B] TEM images . [C] Nano particle size distribution. [D] ICP-OES analysis of percentage of Ni $^{+2}$ doping.

XRD and FT-IR study



Peak	Planes	Peak	Planes
a	HA002	f	HA310
b	HA102	g	HA222
c	HA211	h	HA213
d	HA300	i	HA402
e	HA202	j	HA004

XRD



	Wavenumber (cm^{-1})	Groups
a	470	$\upsilon_2(PO_4^{3-})$
b	550-600	$\upsilon_4(PO_4^{3-})$
d	960	$\upsilon_1(PO_4^{3-})$
e	1020-1120	$\upsilon_3(PO_4^{3-})$
g	3572	$\upsilon_3(OH^-)$
c	860	$\upsilon_2(CO_3^{2-})$
	1470	$\upsilon_3(CO_3^{2-})$

FTIR

Analysis of crystal parameters

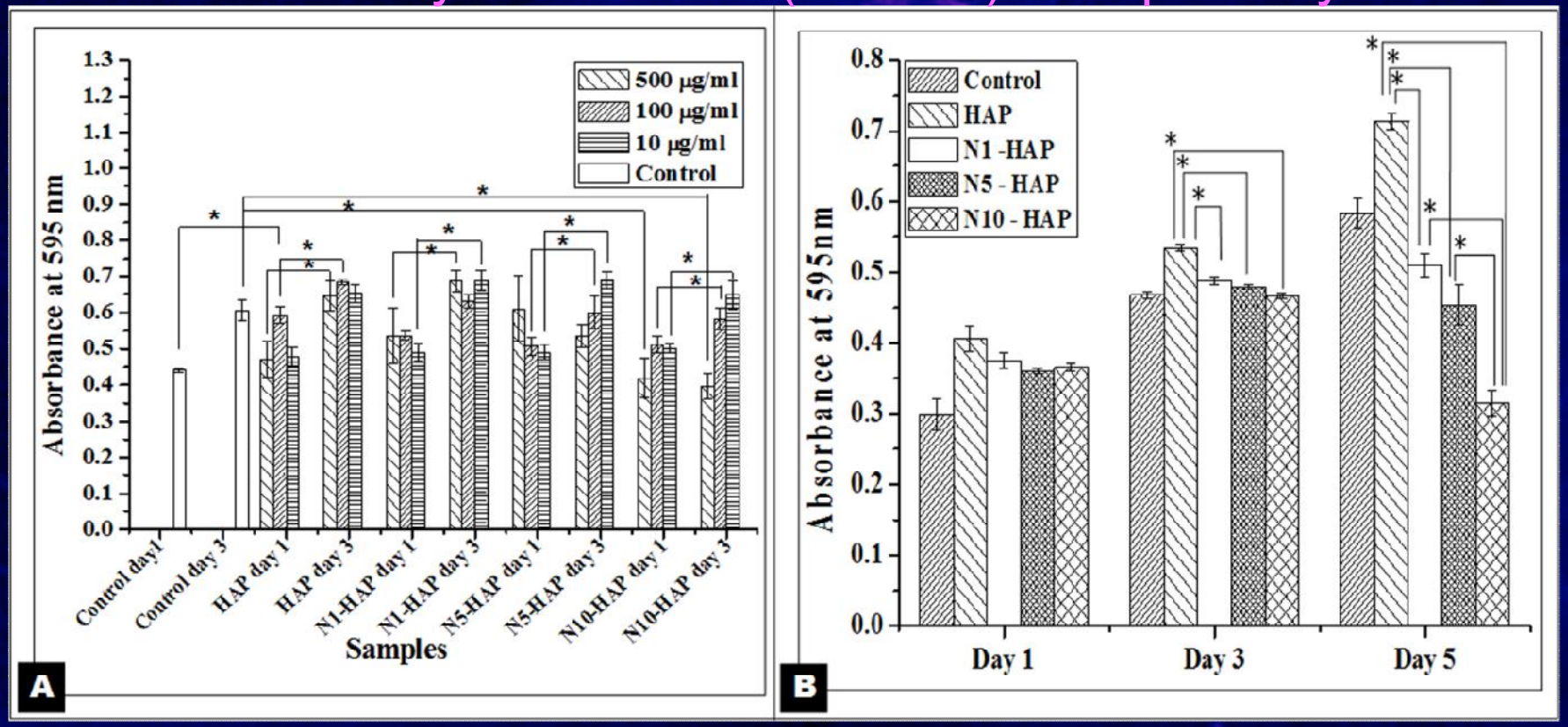
Samples	% Crystallinity	Crystal lattice parameters(A°)		'd' spacing (nm)
		' a '	' c '	
HAP	42.0	9.37	6.88	0.3644
N1-HAP	42.0	9.43	6.90	0.3642
N5-HAP	46.0	9.37	6.88	0.3624
N10-HAP	22.0	9.43	6.90	0.3578

Crystal parameters of nHAp and Ni2+ doped nHAp. Percentage crystallinity and ' d' spacing were calculated using the XRD and TEM diffraction data corresponding to 002 plane. For the analysis of crystal lattice parameters, ' a' and ' c', 300 and 002 planes were considered.

Analysis of surface area, zeta potential and protein adsorption

Samples	Surface area ($m^2 g^{-1}$)	Pore volume ($cm^3 g^{-1}$)	Zeta potential (mV)	Protein adsorption (µg/100mg)
HAP	109.1	0.041	- 3.5	881
N1-HAP	107.6	0.040	- 1.0	885
N5-HAP	111.7	0.046	3.2	873
N10-HAP	83.2	0.034	1.1	878

Study of bone cell (MG -63) compatibility

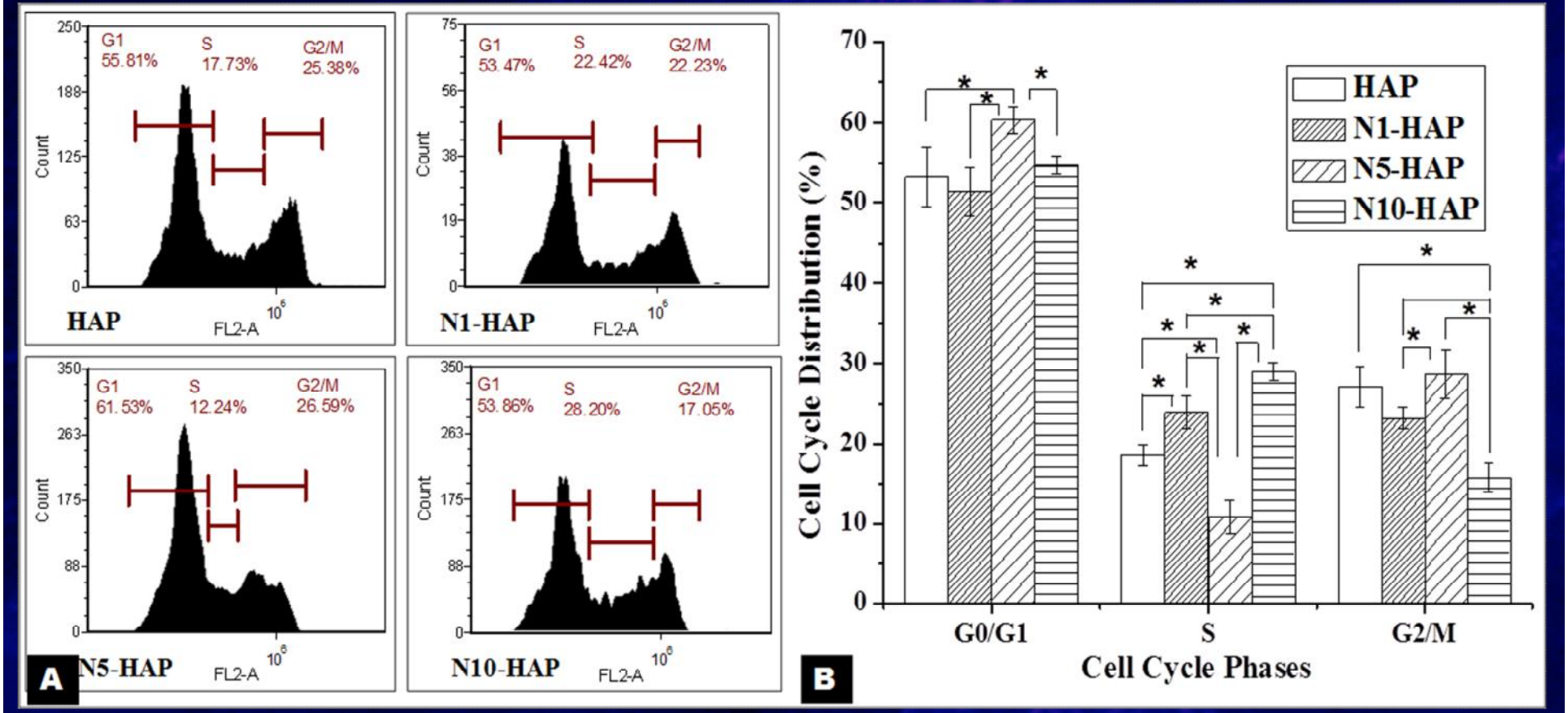


Cell viability and cell proliferation study by MTT assay. [A] Cells were treated with Ni^{+2} doped nHAp at concentrations of 500 mg/ml, 100 mg/ml and 10 mg/ml. [B] Cells were treated with Ni^{+2} doped nHAp at a concentration of 100 mg/ml for 5 days.

Flow cytometry based live - dead assay: Percentage of live cells

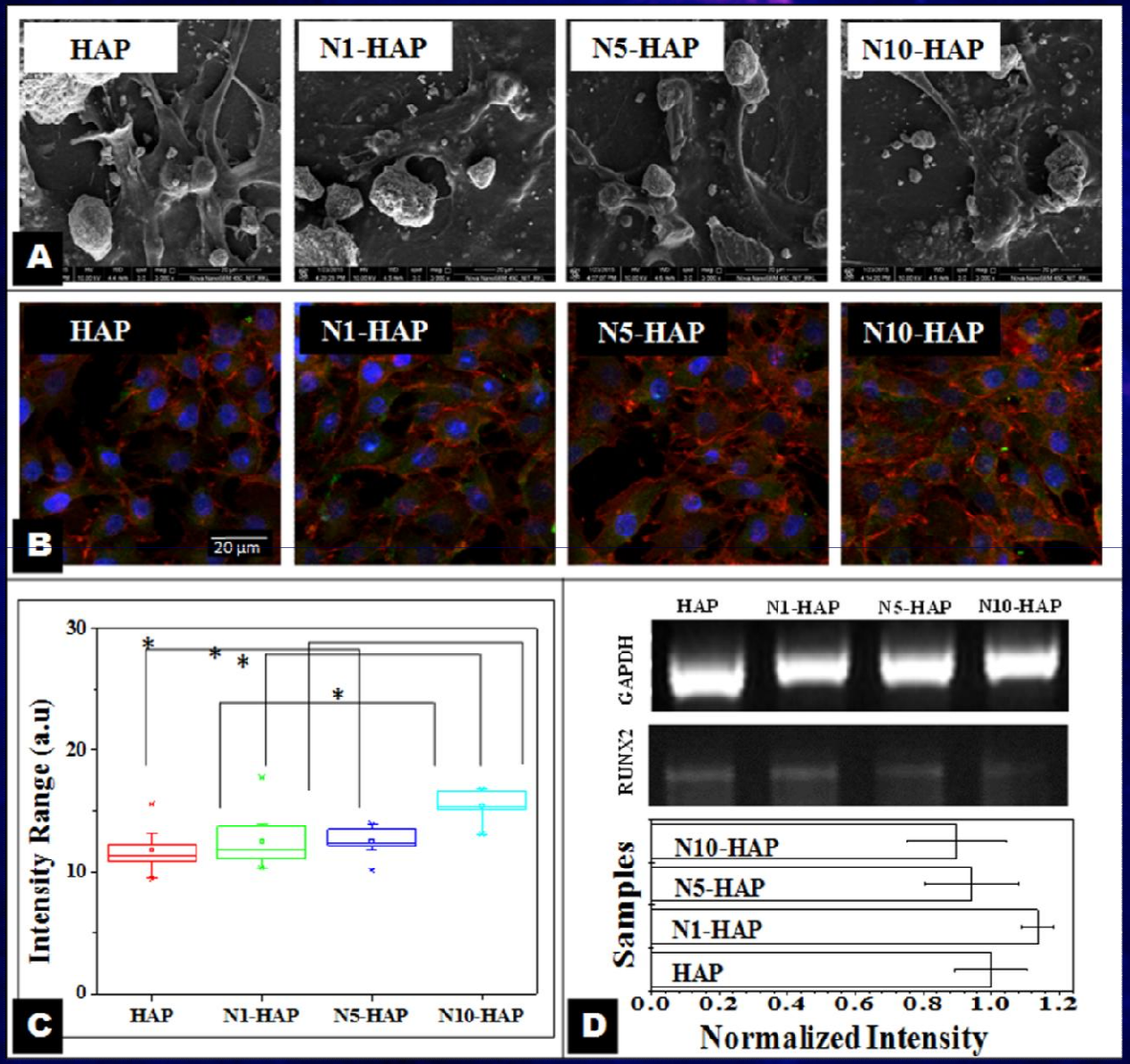
	HAP	N1-HAP	N5-HAP	N10-HAP	Control
Day 1	94.9	93.4	92.7	89.9	94.9
Day 3	91.7	93.3	92.9	94.5	94.2
Day 5	92.1	92.1	91.1	92.3	95.1

Cell cycle analysis



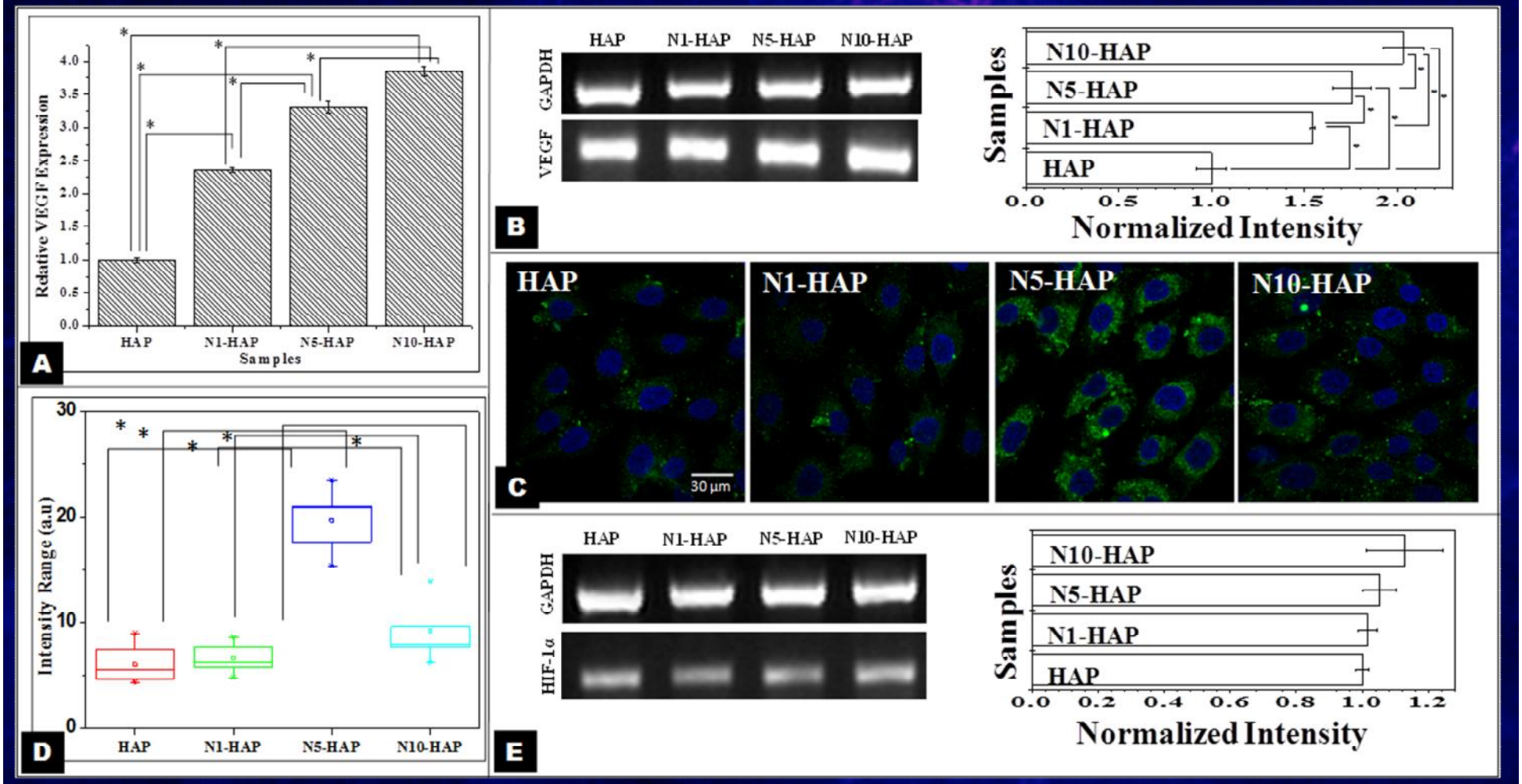
Study of the cell cycle after 24 h of treatment. Cells were treated with Ni2+ doped nHAp at a concentration of 100 mg / ml for a period of 24 h. [A] Representative cell cycle histograms for each sample were presented. [B] Quantitative analysis of cell cycle data.

Study of the osteoconductive property



SEM [A] and confocal [B] images of cells cultured in presence of doped nHAp (100 mg / ml) at day 3. Red [F actin], blue [Hoechst] and green [Runx2]. [C] Quantitative image analysis of Runx2 expression.

Study of the proangiogenic property



[A] Analysis of VEGF expression by ELISA. [B] Study of VEGF mRNA expression by RT-PCR. [C] Study of HIF-1a expression by immuno-cytochemistry. [red (F actin), blue (Hoechst) and green (HIF-1a)]. [D] Quantitative image analysis of HIF-1a expression. . [E] Study of HIF-1a mRNA expression by RT-PCR.

Conclusions

Doping of Ni $^{2+}$ in Modification of nano hydroxyapatite by Ni^{2+} doping and by gumtragacant conjugation improve its

- Osteo-conductive property
- Osteogenic property
- Proangiogenic property

Publications

[1] S. Pandey, K. Senthilguru, K. Uvanesh, Sai S. Sagiri, B. Behera, N. Babu, Mrinanl K. Bhattacharyya, K. Pal, **I. Banerjee**, Natural gum modified emulsion gel as single carrier for the oral delivery of probiotic-drug combination, **International journal of biological macromolecules**, **(2016) In press**. **(Impact factor: 3.13)**

[2] T. Agarwal, R. Narayan, S. Maji, S. Behera, S. Kulanthaivel, T.K. Maiti, **I. Banerjee**, K.Pal, S.Giri, Gelatin/Carboxymethyl chitosan based scaffolds for dermal tissue engineering applications, **International journal of biological macromolecules, (2016) In press. (Impact factor: 3.13)**

[3] S. Kulanthaivel, B. Roy, T. Agarwal, S. Giri, K. Pramanik, K. Pal, S.S. Ray, T.K. Maiti, **I. Banerjee,** Cobalt doped proangiogenic hydroxyapatite for bone tissue engineering application, **Materials Science and Engineering: C, 58 (2016) 648-658. (Impact factor: 3.42)**

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[5] B.A. Priya, K. Senthilguru, T. Agarwal, S.G.H. Narayana, S. Giri, K. Pramanik, K. Pal, I. Banerjee, Nickel doped nanohydroxyapatite: vascular endothelial growth factor inducing biomaterial for bone tissue engineering, RSC Advances, 5 (2015) 72515-72528. (Impact factor: 3.29)

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[8] V.K Singh, I.Banerjee, K.Pramanik, M.K Bhattacharya, K.Pal, Preparation and characterization of novel carbopol based bigels for topical delivery of metronidazole. Materials Science and Engineering: C, (2014). (Impact factor: 3.42)

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Student's contribution

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Thank You