

**Effects of Hydroxyapatite-Iron Oxide Nanocomposite
Against Human Glioblastoma Cells**

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THESIS

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This thesis is dedicated to my parents, Bożena and Richard Pernal, for their unwavering love and support throughout my academic career.

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TABLE OF CONTENTS

I.	INTRODUCTION	1
A.	Background	1
B.	Statement of Problem.....	4
C.	Utilizing Hydroxyapatite	6
D.	Anisotropy of Cellular Cytoskeleton	7
E.	Significance of the Study	7
II.	METHODS	9
A.	Iron Oxide Nanoparticle Synthesis	9
B.	Hydroxyapatite Synthesis	9
C.	Hydroxyapatite-Iron Oxide Nanoparticle Synthesis.....	10
D.	Transmission and Scanning Electron Microscopy.....	12
E.	X-Ray Diffraction, Crystallinity, and Iron Oxide Composition	12
F.	Dynamic Light Scattering and Zeta Potential	13
G.	SQUID Magnetometry.....	13
H.	Cell Cultures	14
I.	Cytotoxicity of Nanoparticles	15
J.	Uptake and Fluorescent Imaging and Processing	15
K.	Spheroid Migration Assay	17
L.	Actin Cytoskeleton Analysis.....	17
M.	Statistical Evaluation	18
III.	RESULTS.....	19

A.	Scanning and Transmission Electron Microscopy.....	19
B.	Nanocomposite Size and X-Ray Diffraction	21
C.	Magnetic Hysteresis of HAP-IONS	26
D.	Nanoparticle Cytotoxicity	27
E.	Migration of E297 Spheroids.....	32
F.	Uptake of Nanoparticles	36
G.	Change in the Anisotropy of the Actin Cytoskeleton	42
IV.	DISCUSSION.....	46
V.	REFERENCES	52
VI.	APPENDICES	61
A.	MATLAB CODE.....	61
1.	Localization.m	61
2.	LocalizationBlackfromRB.m	62
3.	LocalizationBlackfromGreen.m.....	63
4.	LocalizationGreen.m.....	64
VII.	VITA.....	66

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
TABLE I. FINAL CONCENTRATION OF MATERIALS IN NANOCOMPOSITES	11
TABLE II. MATERIAL PROPERTIES OF NANOCOMPOSITES	24
TABLE III. CRYSTALLITE SIZES OF HAP AND MAGNETITE IN NANOCOMPOSITES...	24

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
Figure 1. Magnetic field versus magnetization of superparamagnetism, paramagnetism and ferromagnetism	3
Figure 2. Average number of IONs uptaken by both primary and cancer cell lines after 24-hour treatment.	5
Figure 3. Scanning electron micrographs of IONs and HAPs	19
Figure 4. Scanning electron micrographs of HAP-IONs	20
Figure 5. Transmission electron micrographs of IONs, HAPs, and HAP-IONs	21
Figure 6. X-ray diffractogram of nanocomposite materials.....	23
Figure 7. Zeta potential measurements of nanocomposite materials.....	26
Figure 8. Hysteresis curve of 1CsHAP and 10CsHAP.....	27
Figure 9. Cytotoxicity in human mesenchymal stem cells compared to IONs, MBs, HAP, and HAP-ION nanocomposites	28
Figure 10. Cytotoxicity in U87 human glioblastoma compared to IONs, MBs, HAP, and HAP-ION nanocomposites.....	29
Figure 11. Cytotoxicity in E297 primary patient glioblastoma compared to IONs, MBs, HAP, and HAP-ION nanocomposites	31
Figure 12. Average migration of E297 spheroids.....	33
Figure 13. E297 spheroid migrations in ION and MB treatments.....	34
Figure 14. E297 spheroid migrations in nanocomposite treatments.....	35
Figure 15. Uptake of nanocomposites in various cell lines.	37
Figure 16. Fluorescence images of MSC treated with nanocomposites	38
Figure 17. Fluorescence images of U87 treated with nanocomposites.....	39
Figure 18. Fluorescence images of E297 treated with nanocomposites	40

Figure 19. Fluorescence images of MSC and U87 with ION and MB	41
Figure 20. Change in the actin cytoskeleton anisotropy versus control in MSC	43
Figure 21. Change in the actin cytoskeleton anisotropy versus control in U87	44
Figure 22. Change in the actin cytoskeleton anisotropy versus control in E297	45

LIST OF ABBREVIATIONS

10CnHAP	10 mL of ION with no-base-added hydroxyapatite composite
10CsHAP	10 mL of ION with standard hydroxyapatite composite
1CnHAP	1 mL of ION with no-base-added hydroxyapatite composite
1CsHAP	1 mL of ION with standard hydroxyapatite composite
DLS	Dynamic light scattering
E297	Human patient glioblastoma
HAP	Hydroxyapatite
HAP-IONs	Hydroxyapatite iron-oxide nanocomposite
hMSC	Human mesenchymal stem cells
IONs	Iron oxide nanoparticles
K7M2	Mouse osteosarcoma
Kidney F	Kidney fibroblasts
Lung F	Lung fibroblasts
MB	Magnetic Beads
MH	Magnetic hyperthermia
nHAP	No-base-added hydroxyapatite
SEM	Scanning electron microscopy
sHAP	Standard hydroxyapatite
SPIONs	Superparamagnetic iron oxide nanoparticles
TEM	Transmission electron microscopy
U87	Human glioblastoma
XRD	X-ray diffraction

SUMMARY

Cancer is the second leading cause of death in the United States (1) and few treatments offer a panacea for all the various types of cancer that exist. Certain superparamagnetic iron oxide nanoparticles (SPIONs) are FDA-approved as contrast agents in magnetic resonance imaging while the potential applications for SPIONs are numerous. One such application is magnetic hyperthermia (MH) where alternating magnetic fields applied to SPIONs generate heat (2). MH in treating cancer would cause cancer cells to deteriorate from the inside without the use of strong chemotherapeutics. SPION effectiveness is hindered by the low uptake in cancer cells and the generation of reactive oxygen species that cause harm to the healthy cells in the body (3).

In early studies, healthy, primary mouse kidney and lung fibroblasts had an increased uptake of SPIONs versus human brain cancer cells (E297 and U87) and mouse osteosarcomas (K7M2.) Hydroxyapatite (HAP), the primary ceramic in our bones (4), offers a solution to SPION delivery. HAP particles are commended as a biomaterial for their biodegradability and biocompatibility (5), and their use as a nonviral transfection agent (6). Dispersing SPIONS in HAP nanoparticles could increase the uptake in cancer cells and minimize the risk to healthy cells.

The goal of this work is the construction and characterization of a hydroxyapatite-iron oxide nanocomposite (HAP-ION) usable as a device for magnetic hyperthermia, minimizing the risk to healthy cells and increasing their uptake in cancer cells. HAP-IONs retain the superparamagnetic nature of SPIONs, have increased uptake in cancer cells versus their SPION counterparts, reduce cancer cell viability and primary cancer spheroid migration, and maintain the viability of healthy human mesenchymal stem cells (hMSCs). Further analysis of actin cytoskeleton revealed that healthy hMSCs have a reduced anisotropy in their cytoskeleton

arrangement after the uptake of SPIONs, while primary cancerous cells have an increased anisotropy in cytoskeleton arrangement after the uptake of SPIONs. Nanocomposites of hydroxyapatite and SPIONs open a new avenue to cancer therapies that utilize MH.

I. INTRODUCTION

A. Background

Cancer is the second leading cause of death in the United States (1) and few treatments offer a panacea for all the various types of cancer that exist. Current cancer therapies include surgery, chemotherapy and radiation therapy (7) and more recent therapies utilize targeted therapies that interfere with cancer cell progression (8) and nanotechnology that can detect and treat early cancerous tissues (9).

Certain superparamagnetic iron oxide nanoparticles (SPIONs) are FDA-approved as contrast agents in magnetic resonance imaging while the potential applications for SPIONs are numerous. One such application is magnetic hyperthermia (MH) where alternating magnetic fields are applied to SPIONs to generate heat and cause thermal inactivation of proteins or necrosis from within a cell (2,10,11). Heat is generated through multiple mechanisms: (i) hysteresis losses; (ii) Néel relaxation; (iii) Brownian relaxation; and (iv) frictional losses. Hysteresis loss accounts for the energy lost to heat after the reversal of magnetization in a material (12). The area under the curve of a hysteresis loop defines the energy lost during one complete cycle of magnetization. Néel relaxation refers to the reorientation of the internal magnetic moment of the nanoparticle, while the Brownian relaxation refers to the reorientation of the whole particles (13). Finally, frictional loss is the heat generated from the friction of the particles moving within a medium (14). Frictional loss does not require a nanoparticle to be superparamagnetic, but only to have a magnetic moment and acquire kinetic energy in the magnetic field.

Magnetism arises from the organization of unpaired electrons, where the electron spins orient with atoms of another region, generating a net magnetic moment. Materials that

spontaneously align to a magnetic field have magnetic domains, a specific portion of a material has a uniform direction in its magnetization. Superparamagnetism is a type of magnetism where a material becomes magnetic in the presence of a magnet. Superparamagnetism differs from ferromagnetism in that ferromagnetic materials retain a portion of the magnetization after a magnetic field has been applied and removed, while a superparamagnetic material does not retain its magnetization, as demonstrated in figure 1. Ferromagnetic particles have a multi-domain structure, where each domain can have its own magnetization (15) while superparamagnetic particles are considered single domain particles (16). As a magnetic field is removed, the ferromagnetic material cannot maintain the orientation of the dipoles in every domain, and the magnetization is reduced and retained. Superparamagnetic particles are size dependent and require a finite size to match the magnetic domain (15). Paramagnetic materials respond by aligning to external magnetic fields similar to superparamagnetic materials but do not have magnetic domains and do not spontaneously align. In paramagnetic materials, magnetic dipoles are not pointed in the same direction, negating a portion of the total magnetization

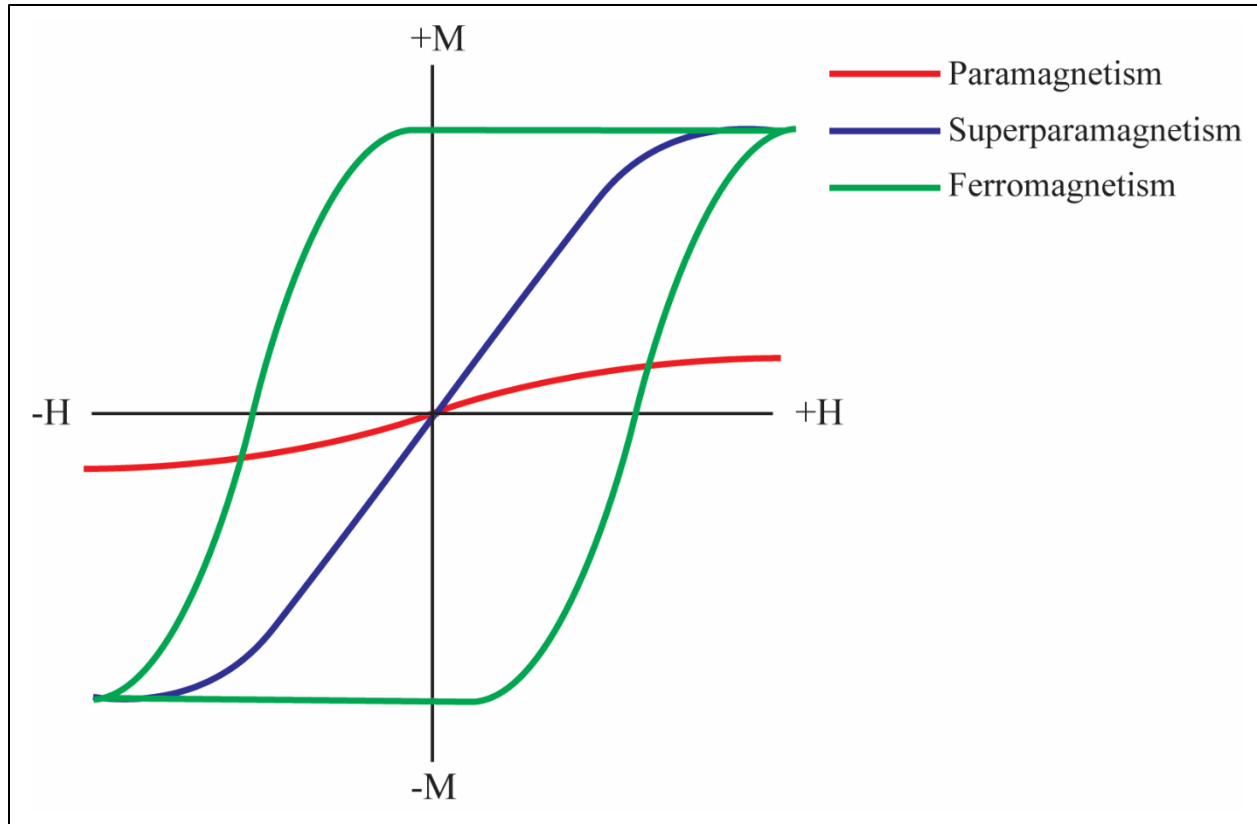


Figure 1. Magnetic field versus magnetization of superparamagnetism, paramagnetism and ferromagnetism

During MH, SPIONs are injected into a tumor or localized at a tumor using an external magnet, then the region of interest is placed in an alternating magnetic field with a certain frequency and amplitude (17). As the frequency of the alternating magnetic field increases, the magnetic moment changes rapidly and releases heat. To minimize patient discomfort and maintain MH effectiveness, the magnetic field alternates at 100 kHz with a strength of 15 kA/m (18). In clinical trials, patients with glioblastoma multiforme were injected with SPIONs into the tumor area with six sessions of treatment. The median survival time for patients treated with SPIONs and MH was 13.4 months compared to 6.2 months in the control group and observed as significant (18).

MH was once considered the fourth leg of cancer therapy but has fallen into decline (19). In early experimentation, it was believed that cancer cells were more sensitive to heat but in fact that this varies among cells and tissues (20). MH with IONs would be more beneficial in conjunction with current cancer treatment therapies. IONs are superparamagnetic and can be localized to specific regions. Localization would reduce systemic toxicity by maintaining the treatment to a specific tissue unlike conventional chemotherapy that utilizes systemic drug distributions or radiation therapy that bombards large sections of tissue with radioactivity. Reducing the occurrence of systemic toxicity in cancer treatment therapies is possible through IONs, which can be effectively localized and concentrated to targeted regions, and treated with MH.

B. Statement of Problem

To effectively cause thermal inactivation and necrosis in cancer cells with MH, SPIONs need to be uptaken by the targeted tumor cells. Preliminary studies demonstrated that primary mouse lung fibroblasts (Lung F) and primary mouse kidney fibroblasts (Kidney F) had a greater uptake of iron oxide nanoparticles (IONs) than mouse osteosarcoma (K7M2), human glioblastoma cultured cell line (U87), and a primary patient human glioblastoma cell line (E297), as seen in figure 2. IONs would be an ineffective cancer treatment because IONs are uptaken in greater amounts in the healthy primary cells than the cancers cells, resulting in a greater degree of cell death among the healthy non-cancerous cells than cancer cells. Therefore, the risk of harming healthy cells needs to be minimized and the uptake of SPIONs in cancer cells needs to be increased.

The increased ION uptake in lung fibroblasts versus kidney fibroblasts is disconcerting but expected. Lung tissue is regularly a site of accumulation for nanoparticles such as gold (21), carbon nanotubes (22), and IONs (23), only surpassed by the accumulation in the liver and the spleen. Jain *et al.* demonstrated that lung tissue had greater iron levels than kidney tissue within the first 24 hours following intravenous injection of IONs (23). Liver and spleen are expected to have larger amounts of ION concentration as both organ systems are involved in the reticuloendothelial system (24,25), and recycling and metabolizing iron (26).

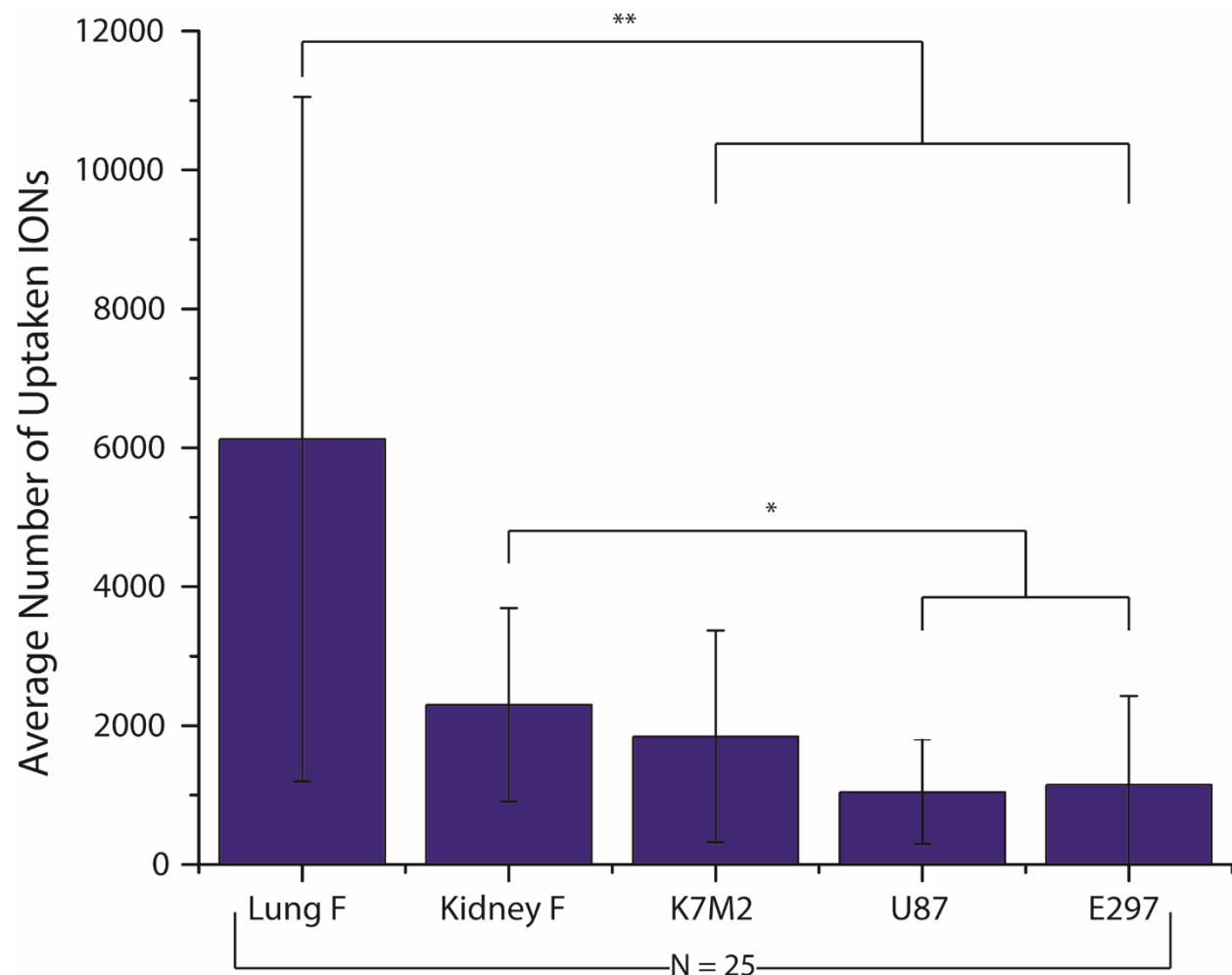


Figure 2. Average number of IONs uptaken by both primary and cancer cell lines after 24-hour treatment. IONs were stained using Prussian blue and uptake determined using MATLAB code, Localization.m (Appendix 1.1). * refers to $P < 0.01$; and ** refers to $P < 0.0005$.

C. Utilizing Hydroxyapatite

Hydroxyapatite (HAP), the primary ceramic in our bones (4), offers a solution to the problem of a greater ION uptake by healthy cells rather than the cancer cells. HAP particles are commended as a biomaterial for their biodegradability and biocompatibility (5,27), as well as their use as a nonviral transfection agent (6,28). Previous studies on HAP nanoparticles demonstrate their ability to be an effective delivery vehicle of antibiotics in treating osteomyelitis (29,30), and as a vehicle with tunable release kinetics (5,6).

HAP may have a selective cytotoxicity against cancer and healthy cells. HAP, for example, imparted a cytotoxic effect on hepatoma cells and imparted no cytotoxic effect on healthy hepatocytes (31). HAP appears to localize around the endoplasmic reticulum interfering with protein synthesis and as the cancer cell endocytose more material, the effect becomes more pronounced (32). In the same study, Han *et al* demonstrated that gastric cancer, liver cancer, and osteosarcomas cell lines experienced a greater degree of proliferation inhibition than in healthy cell lines of hepatocytes, lung fibroblasts, and keratinocytes (32). HAP is readily uptaken by cells (5,33). Size determines uptake of the HAP particles (34) and HAP particles are predominantly uptaken using micropinocytosis (35). Combining the applications of IONs and effects of HAP into a nanocomposite offers a method to increase uptake in cancerous cells and minimize the risk to healthy cells.

D. Anisotropy of Cellular Cytoskeleton

Anisotropy is the property of being directionally dependent; in the context of a cell structure, it can define if cytoskeletal fibrils are highly ordered or disorganized. The cytoskeleton governs the shape of the cell, provides a scaffold for organelles, the uptake of materials and cell signaling (36,37). It is also involved in cancer cell metastasis and invasion (38,39). Alzheimer's disease demonstrates that alterations in the cytoskeleton affect the characteristics of the cell, in particular with the disintegration of microtubules leading to neurodegeneration (40). Creekmore *et al* demonstrated that cytoskeletal disorganization correlated with a progression from non-tumorigenic to aggressive, malignant phenotype in mouse ovarian epithelial cells (41).

Elucidating the organization and structure of the cytoskeleton after nanoparticle uptake can further our understanding of how a cellular system responds to foreign material and to what extent does a nanoparticle system needs to be modified to increase uptake (42–44). Following uptake of IONS, microtubules remodel, inducing increased endothelial cell permeability, and that reactive oxygen species control the extent of the remodeling (45). Unfortunately, no studies have been conducted analyzing the changes in the actin cytoskeleton after the uptake of ION.

E. Significance of the Study

For this thesis, I present the construction and characterization of a hydroxyapatite-iron oxide nanocomposite (HAP-IONs). HAP-IONs retain their superparamagnetic ability, have increased uptake in cancer cells versus their ION counterparts, reduce cancer cell viability and decrease cancer spheroid migration, and maintain the viability of healthy human mesenchymal

stem cells (MSCs). Further analysis of actin cytoskeleton revealed that healthy MSCs have a reduced anisotropy and less order in their cytoskeleton arrangement after treatment with IONs, causing an increase in the cell permeability of IONs; however, primary cancerous cells have an increased anisotropy and more order in cytoskeleton arrangement after treatment with IONs, causing a decrease in the cell permeability of IONs. Nanocomposites of hydroxyapatite and IONs open a new avenue to cancer therapies that utilize MH. HAP-IONs are biocompatible materials can be localized to specific regions decreasing systemic toxicity, leave no radioactive or heavy metal material, and preferentially harm cancer cells while sustaining healthy cell viability.

II. METHODS

A. Iron Oxide Nanoparticle Synthesis

Iron oxide nanoparticles (IONs) were synthesized using a molar ratio of 2:1 $\text{Fe}^{3+}:\text{Fe}^{2+}$. Iron (III) chloride hexahydrate (Alfa Aesar, Haverhill MA) and iron (II) chloride tetrahydrate (Alfa Aesar, Haverhill MA) salts were dissolved in 2% ammonium hydroxide (Sigma Aldrich, St. Louis MO) v/v solution in water creating a solution with a final concentration of 10 mM ferric ions and 5 mM ferrous ions. The 100 mL iron solution was added 1 drop per second into a 400 mL solution of 0.1% Triton X-100 (Arcos Organics, New Jersey) and 1 M sodium hydroxide (Fisher Scientific, Hampton NH) at 80°C, and mixed vigorously at 1800 rpm for 1 hour, creating the ION stock solution. For individual assays, ION stock solution was diluted with deionized water to a 5 mg/mL standard. Final concentrations of nanoparticle materials are defined in Table 1.

As an industry standard, Magnetic Beads (MB) from Pulse Therapeutics (St. Louis, MO) were diluted with deionized water to 5 mg/mL. MBs are iron oxide nanoparticles with a PEG coating. MBs are used to determine the effectiveness and efficacy of a commercially available SPION against that of the synthesized IONs.

B. Hydroxyapatite Synthesis

Two forms of hydroxyapatites were synthesized: (i) no-base-added HAP henceforth known as nHAP; and (ii) standard HAP henceforth known as sHAP.

nHAP was synthesized using a modified procedure of Andronescu *et al* (46). In short, 100 mL of 0.1 M calcium nitrate tetrahydrate (Fisher Scientific, Hampton NH) and 100 mL of 0.06 M

ammonium phosphate monobasic (Fisher Scientific, Hampton NH) were mixed together dropwise adding the calcium solution into a mixing phosphate solution at 13.33 mL per minute for 1 hour.

sHAP was synthesized using the procedure described earlier (29). In short, 100 mL total of 0.1 M calcium nitrate tetrahydrate with 12 mL ammonium hydroxide (Sigma Aldrich, St. Louis MO), and 100 mL of 0.06 M ammonium phosphate monobasic with 6 mL ammonium hydroxide were mixed together dropwise while adding the calcium solution into a mixing phosphate solution at 13.33 mL per minute for 1 hour.

Both HAP materials underwent the same wash procedure after synthesis. Solution was poured into Falcon tubes and centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded, and 35mL of deionized water was poured into each tube, vortexed, then centrifuged again at 3500 rpm for 5 minutes. This procedure was repeated once more with 100% ethanol instead of deionized water. Following centrifugation, samples were dried for 48 hours at 37°C in an Incu-Shaker (Benchmark Scientific, South Plainfield, NJ).

sHAP follows a standard procedure of synthesizing HAP, while nHAP follows a procedure lacking the additional ammonium hydroxide.

C. Hydroxyapatite-Iron Oxide Nanoparticle Synthesis

Four hydroxyapatite-iron oxide nanocomposites of two varying ION concentrations in both nHAP and sHAP were created by adding a volume of ION stock solution into the calcium solution. Final concentrations of each material is listed in Table 1.

Nanocomposite with 1 mL ION stock solution was added to the 100mL 0.1 M calcium solution of either HAP before adding the calcium solution dropwise into mixing 100 mL of 0.06

M phosphate solution at 13.33 mL per minute. 1 mL ION added into sHAP or nHAP for the nanocomposite henceforth will be known as 1CsHAP or 1CnHAP, respectively.

Nanocomposite with 10 mL ION stock solution was added to the 100 mL 0.1 M calcium solution of either HAP before adding the calcium solution dropwise into a mixing 100 mL of 0.06 M phosphate solution at 13.33 mL per minute. 10 mL ION added into sHAP or nHAP for the nanocomposite henceforth will be known as 10CsHAP or 10CnHAP, respectively.

Both HAP-ION materials underwent the same wash procedure after synthesis. Solution was poured into Falcon tubes and centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded, and 35mL of deionized water was poured into each tube, vortexed, then centrifuged again at 3500 rpm for 5 minutes. This procedure was repeated once more with 100% ethanol instead of deionized water. Following centrifugation, samples were dried for 48 hours at 37°C in an Incu-Shaker (Benchmark Scientific, South Plainfield, NJ).

TABLE I.

FINAL CONCENTRATIONS OF MATERIALS IN EACH NANOPARTICLE SYSTEM.

Nanoparticle System	Final Concentration of Material				NH ₄ OH Added
	[Fe ³⁺] (mM)	[Fe ²⁺] (mM)	[Ca ²⁺] (mM)	[PO ⁴⁻] (mM)	
ION	5	2.5	0	0	Yes
MB	n/a*	n/a*	0	0	n/a*
nHAP	0	0	50	30	No
sHAP	0	0	50	30	Yes
1CnHAP	0.025	0.0125	50	30	No
1CsHAP	0.025	0.0125	50	30	Yes
10CnHAP	0.25	0.125	50	30	No
10CsHAP	0.25	0.125	50	30	Yes

n/a* refers to not applicable as the material was received from Pulse Therapeutics (St. Louis, MO).

D. Transmission and Scanning Electron Microscopy

TEM images were taken with a JEOL JEM 1220 Life Science TEM under the direction of Maheshwar Iyer Adiraj (UIC, Department of Bioengineering). SEM images were taken with a JEOL JSM 6320 FESEM under the direction of Shreya Ghosh (UIC, Department of Bioengineering). The aspect ratio of needle-like particles was measured using MATLAB's (Natick, MA, USA) Image Viewer app. Aspect ratio is defined as the length of the major axis divided by the width of the minor axis. Spheres have an aspect ratio of 1, and rods would have an aspect ratio greater than 1.

E. X-Ray Diffraction, Crystallinity, and Iron Oxide Composition

X-ray diffraction (XRD) was conducted using a Bruker D2 Phaser 2nd Gen. Scans were 10 to 90° with a 0.0012 step for 1 second per step for a total scan time of 2 hours. Samples were dried and prepared as powders. MB did not have an XRD conducted because the material was commercially produced. Crystallinity in HAP materials was calculated using the procedure outlined by Land *et al* (47) using equation (II.1). The degree of crystallinity, X_c , is in relation to the intensity of the (300) reflection, I_{300} , and the intensity of the hollow between (112) and (300) reflections, $V_{112-300}$, which disappears in non-crystalline samples.

$$X_c \approx 1 - \left(\frac{V_{112-300}}{I_{300}} \right) \quad (\text{II.1})$$

Iron oxide phase composition was quantified using the procedure outline by Wonbaek *et al* (48) using equation (II.2). The wt.% of maghemite ($\gamma\text{-Fe}_2\text{O}_3$) can be calculated based on the intensity

fraction of the (511) maghemite peak, $I_{(511)maghemite}$, and the (440) magnetite peak, $I_{(440)magnetite}$. The wt.% of magnetite (Fe_3O_4) was 100% minus the wt.% of maghemite. Data were smoothed using MATLAB before quantification.

$$\left[\frac{I_{(511)maghemite}}{I_{(511)maghemite} + I_{(440)magnetite}} \right] = 1.016 * w_{maghemite} - 0.2371 \quad (II.2)$$

Crystallite size was calculated using Bruker's software, where specific full width at half maximum values were retrieved from the XRD at specific angles. For HAP, (002), (211), and (310) diffraction peaks were used to estimate the crystallite sizes. For ION, (311) and (400) peaks were used.

F. Dynamic Light Scattering and Zeta Potential

Particle size was measured with dynamic light scattering (DLS) and zeta potential was measured using a Malvern Nanoseries ZS (Malvern, UK). Zeta potential sample measurements were pH adjusted between 2 to 10, and scanned 30 times for 10 seconds at each data point. Samples for dynamic light scattering measurements were diluted and sonicated to disperse each sample before each measurement.

G. SQUID Magnetometry

SQUID magnetometry was conducted by Chen Chen (UIC, Department of Mechanical and Industrial Engineering) using a Quantum Design SQUID magnetometer MPMS-5. SQUID

magnetometry was conducted for 1CsHAP and 10CsHAP due to their favorable compositions and cytotoxicity.

H. Cell Cultures

Three mouse cell lines and three human cell lines were used. Primary lung and kidney fibroblasts were created from lung and kidneys isolated from two 8-week old, female C57BL/6J mice. In brief, organs were placed on petri dishes with Hanks' Balanced Salt Solution (Life Technologies, Carlsbad, CA) and continuously minced until a homogenous mixture was achieved. The mixture was poured into centrifuge tubes with 5mg of collagenase type IV (Life Technologies, Carlsbad, CA) and incubated at 37°C. After 30 minutes, the mixture was agitated for 2 minutes before being incubated for 30 minutes again. This procedure was repeated twice. Mixture was poured into petri dishes and grown to confluency before being moved to tissue culture flasks.

K7M2-pCl Neo mouse osteosarcoma was purchased from the American Type Culture Collections (Manassas, VA, USA). U87-MG and E297 human glioblastoma cells were a gift from Dr. Herbert H. Engelhard (UIC, Department of Neurosurgery). Human mesenchymal stem cells (hMSCs) were a gift from Dr. Anne George (UIC, Department of Oral Biology).

All cell lines except for hMSCs were maintained in DMEM with 10% FBS, 1% antibiotic-antimycotic (Life Technologies, Carlsbad CA). Human MSCs were maintained with MesenPro RS medium composed of 10 mL MesenPRO RS growth supplement, 5 mL GlutaMAX-I in 500 mL of MesenPRO RS basal medium. Cell lines were grown to confluency before being plated on 12 mm circular glass cover slips or in 48-well culture plates. Ten-thousand E297 human glioblastoma cells were plated into Corning 96 well ultra-low attachment microplates for 72 hours to form tumor

spheroid. After 72 hours, individual spheroids were plated into 48-well plates coated with 0.1% gelatin and treated with different nanoparticle treatment groups for an additional 72 hours.

I. Cytotoxicity of Nanoparticles

Nanoparticles were diluted into 5 mg/mL standards in water and sonicated for 1 minute. Cell lines were grown in 48-well plates until confluency. Upon confluency, 250 µg/mL or 50 µg/mL of nanoparticles was added to each well. Each plate contained 4 wells of negative control (media alone), 4 wells of sample control (5% v/v water in media), 4 wells each of two 250 µg/mL nanoparticle samples, and 4 wells each of two 50 µg/mL nanoparticles sample.

After 24 hours of incubation, cells were washed with PBS and 275 µL of 1:10 MTT:media v/v added into each well. After 4 hours of incubation at 37 °C, 211 µL of the solution was carefully removed and 125 µL of DMSO added to each well. Plates were placed in a 37 °C incubator shaker at 120 rpm for 30 minutes before measuring the absorbance at 570 nm using the BMG LABTECH FLUOstar Omega microplate reader.

J. Uptake and Fluorescent Imaging and Processing

Cell cultures on cover slips were treated with 250 µg/mL of each nanoparticle system for 24 hours before being washed with PBS and fixed with 4% paraformaldehyde. Preliminary studies utilized Prussian Blue iron staining and Nuclear Fast Red to determine the ION uptake. Fixed cells were stained with 0.5 mL 5% potassium hexacyanoferrate (II) trihydrate w/v and incubated at room temperature 5 minutes to allow Prussian blue pigment to form, then washed with distilled

water, before 0.5 mL of Nuclear Fast Red solution is added for 5 minutes at room temperature. Stained cells were then washed with PBS before being mounted and cured with ProLong Diamond antifade mountant. Presence of blue color (Prussian blue) on slides confirmed the presence of ferric ions and IONs.

HAP containing materials were stained using Lonza's OsteoImage mineralization assay, NucBlue ReadyProbes, and AlexaFluor 568 Phalloidin. In short, cells were fixed using 4% PFA, were washed with wash buffer then stained with 0.2 mL of staining solution of 1:4000 v/v AlexaFluor 568 Phalloidin and 1:100 OsteoImage staining reagent, and one drop of NucBlue added to each well. Fixed cells were incubated at room temperature for 2 hours before being mounted and cured with ProLong Diamond antifade mountant. Samples that did not contain HAP were stained using the same protocol and reagents with the exception of OsteoImage.

Prussian blue and fluorescently stained cells were imaged with a Nikon Eclipse Ti with a DS-Fi2 color camera with a DS-U3 controller and DS-Qi2 CCD camera with X-Cite 120 LED, respectively, using a Plan Apo λ 60x oil objective. Individual cells were cropped from composite images and processed using MATLAB to calculate the amount of ION in uptake images using Localization.m, amount of ION or MB uptake in E297 using LocalizationBlackfromRB.m, amount of ION or MB uptake in U87 and MSC using LocalizationBlackfromGreen.m, and amount of HAP containing materials using LocalizationGreen.m (Appendix 1.1-1.4). In short, images were split into their respective color channels, the blue channel was subtracted from the green channel to reduce noise, before being converted into a black and white based on a specific color with a threshold. The number of pixels that passed the threshold were calculated then multiplied by a factor of 0.05 $\mu\text{m}/\text{pixel}$. This factor was determined by measuring the number of pixels within a scale bar of an image. The result would then be the total micrometers in an image and would be

divided by the size of the nanoparticle sample determined by the dynamic light scattering to calculate the uptake of each nanoparticle.

K. Spheroid Migration Assay

Ten-thousand cells of E297 human glioblastoma cells were plated into Corning 96 well ultra-low attachment microplates for 72 hours for spheroid formation. After 72 hours, spheroids were individually plated into 0.1% gelatin coated 48 well plates and treated with 250 µg/mL of ION, MB, n- and sHAP, and 1Cn- and 1CsHAP for 72 hours. The plates were imaged with Nikon Eclipse Ti with a DS-Fi2 color camera with DS-U3 controller using a Plan Fluor 10x. Images were stitched together using Adobe Photoshop (San Jose, CA, USA) and the length of each cell migration was measured with the Image Viewer app of MATLAB. Cell lengths were calculated by multiplying the pixel distance from the Image Viewer app by a factor of 1.36 µm/pixel. This factor was determined by measuring the numbers of pixels within a scale bare of an image.

L. Actin Cytoskeleton Analysis

Individually cropped fluorescent cell images had their actin cytoskeleton quantified using FibrilTool (49) an ImageJ (50) plug-in. In short, an entire cell was outlined and FibrilTool provides the anisotropy of the actin cytoskeleton in a given region of interest. Cytoskeleton anisotropy was defined as a value between 0 for no order and 1 for perfectly ordered.

M. Statistical Evaluation

Outliers were removed using Tukey Fences (51) where upper and lower bounds are defined as the 1.5 times the interquartile range added to the third quartile and 1.5 times the interquartile range subtracted from the first quartile, respectively. Any value beyond the bounds is considered an outlier. The data expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined using a student's T-test. A value of $P < 0.05$ was considered significant.

III. RESULTS

A. Scanning and Transmission Electron Microscopy

Scanning electron microscopy demonstrated a heterogeneity in the nanocomposite systems, as seen in figures 3 and 4. Figure 3A demonstrates the nanoscale size of the IONs. The nHAP and sHAP systems differed slightly in their overall particle shapes, as seen in figures 3B and 3C, respectively. nHAP appears to be more spherical while sHAP appears to be more needle-like. The nanocomposite materials displayed similar qualities to each other, as seen in figure 5 and were dominated by the morphological properties of the dominant phase, HAP. 1CnHAP, 1CsHAP, and 10CnHAP appear to be more spherical (figure 4A, 4B, 4C, respectively), while 10CsHAP appears to be more needle-like (figure 4D). MB was not imaged using TEM because the material was received from Pulse Therapeutics.

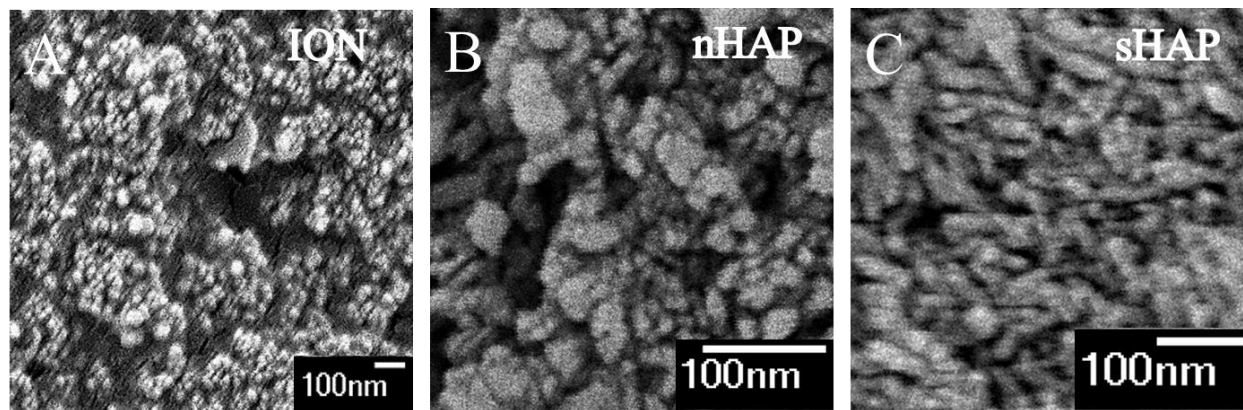


Figure 3. Scanning electron micrographs of: A) IONs; B) nHAP; and C) sHAP.

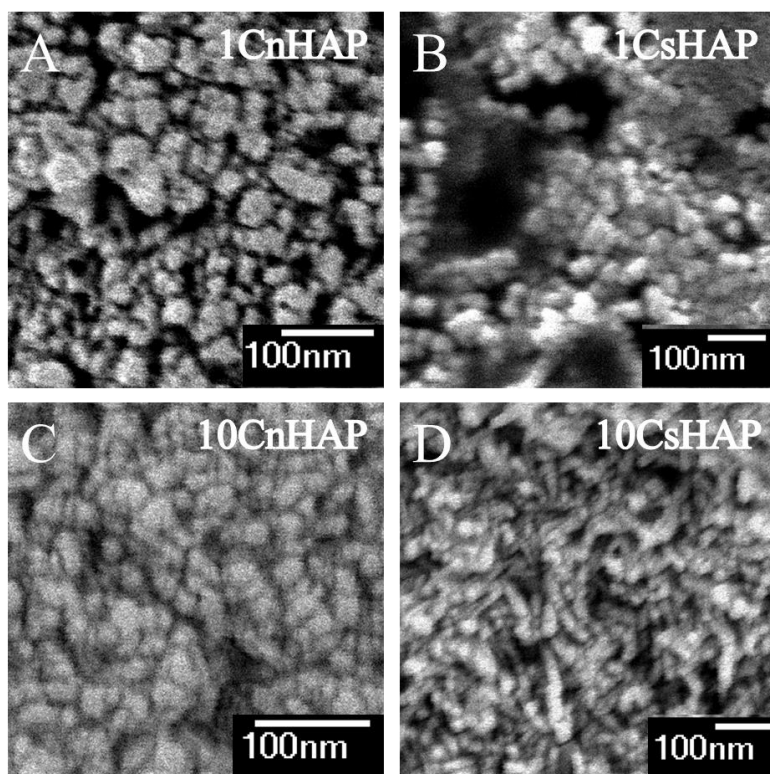


Figure 4. Scanning electron micrographs of: A) 1CnHAP; B) 1CsHAP; C) 10CnHAP; and D) 10CsHAP.

Transmission electron microscopy results differed from scanning electron microscopy. IONs maintained a spherical shape, but the nanocomposites and HAPs appeared more needle-like, as seen in figure 5. SEM and TEM images confirmed that the nanoparticles are indeed nanosized and appear to agglomerate. 10CnHAP was not imaged using TEM because the material was not prepared correctly for imaging. MB was not imaged using TEM since the material was received from Pulse Therapeutics.

The aspect ratio of ION was 1.11 demonstrating a circular shape with slight distortions along the major axis. The aspect ratio of nHAP was 1.65, demonstrating a rod or needle-like quality. sHAP has an aspect ratio of 3.55 and can be considered rod-like. Needle and spherical particles were present in 1CnHAP, with an aspect ratio of 1.68. 1CSHAP was more spherical than

sHAP alone with an aspect ratio of 1.83. 10CsHAP had the most needle structure with an aspect ratio of 3.89.

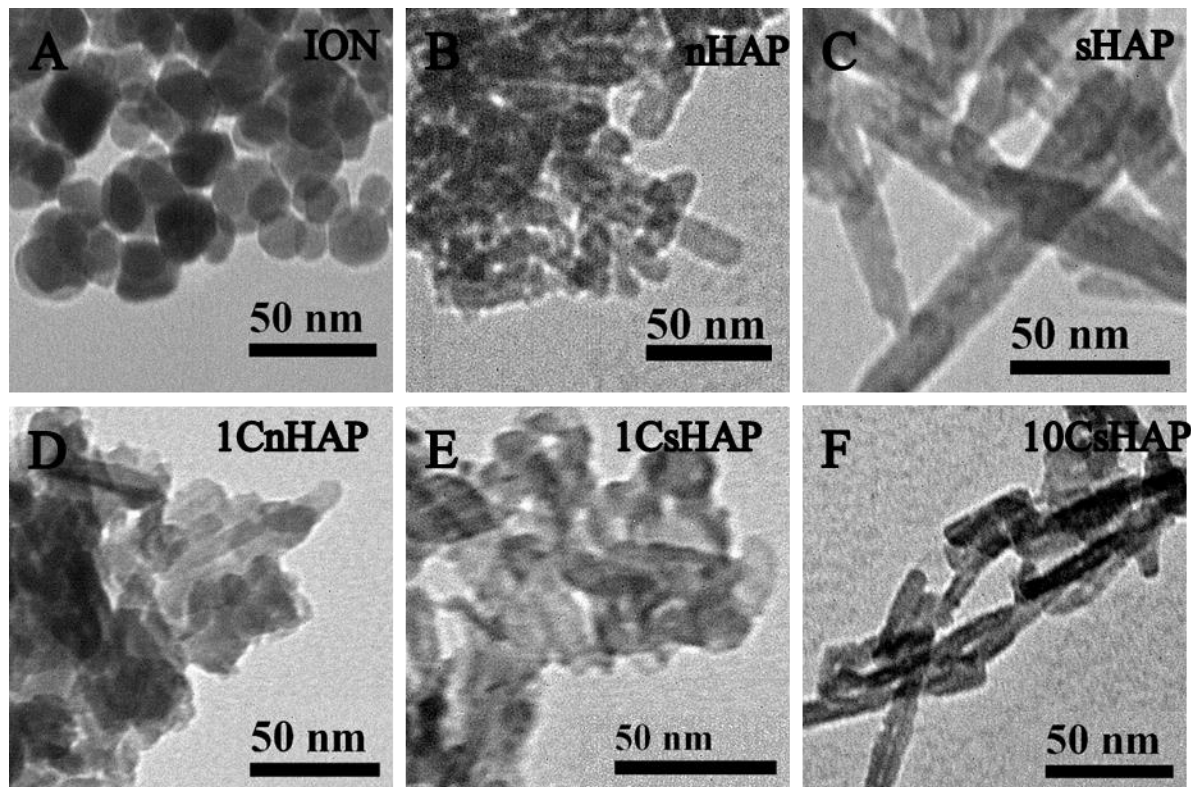


Figure 5. Transmission electron micrographs of: A) IONs; B) nHAP; C) sHAP; D) 1CnHAP; E) 1CsHAP; and F) 10CsHAP.

B. Nanocomposite Size and X-Ray Diffraction

As seen in Table II, the diameter of the nanoparticles increases when HAP and ION are formed into a composite, and increasing the ION concentration from 1 to 10 % reduces the size of

the HAP-ION particles (refer Table I for material concentrations.) The HAP crystallinity of the HAP-containing systems did not follow any linear trends. Increasing the ION concentrations in HAP decreased the crystallinity for 1C-HAPs, but increased the crystallinity for the 10C-HAPs. The material nHAP had increased crystallinity versus sHAP. It is uncertain as to why there was an increase in the HAP crystallinity in the HAP-IONs. The added ION concentration should decrease the crystallinity of HAP, as it is essentially an impurity. ION can have an effect on chemical species involved in precipitation of HAP. ION might consume some of the free hydroxyls through hydration effects and thus lower the supersaturation. As a result, HAP would have slower crystallization and higher crystallinity. The addition of ammonium hydroxide into the synthesis of sHAP-containing HAP-IONs lowered the crystallinity of the HAP because of the higher supersaturation. The composition of the ION in each NP system varied in composition of magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$). There is no linear trend between the magnetite/maghemite composition and the addition of HAP into the system. ION, 1CsHAP, and 10CnHAP had a majority of magnetite in their iron oxide composition, while 1CnAP and 10CsHAP had a majority of maghemite.

Crystallite sizes of the various materials were measured as seen in Table III. Magnetite crystallites decreased in size with increasing ION concentration. ION crystal planes were difficult to determine for HAP-IONs and were left indeterminate. Crystallite sizes containing sHAP were smaller than their nHAP counterparts. As the supersaturation increases, the resulting crystal size is smaller. Figure 6 displays the X-ray diffractograms of the nanocomposite materials.

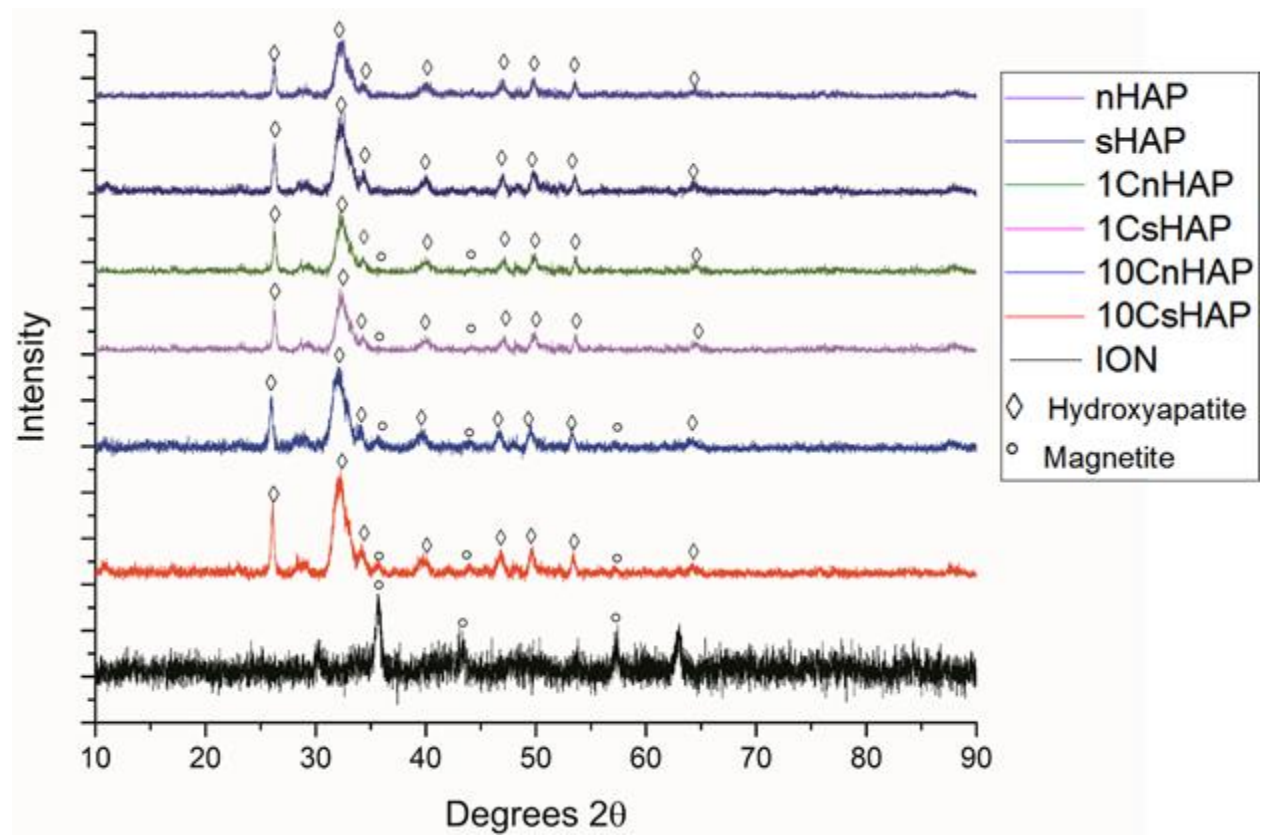


Figure 6. X-ray diffractogram of nanocomposite materials.

TABLE II.

MATERIAL PROPERTIES OF THE NANOCOMPOSITES

Nanoparticle System	Material Properties		
	Diameter (nm)	HAP Crystallinity (%)	Fe ₃ O ₄ / γ -Fe ₂ O ₃ (%/%)
ION	74.79 $\pm$ 9.430	n/a	57.5 / 42.3
MB	33.21 $\pm$ 18.07	n/a	n/a
nHAP	296.9 $\pm$ 30.80	32.9	n/a
sHAP	314.0 $\pm$ 39.70	32.0	n/a
1CnHAP	523.5 $\pm$ 60.12	23.5	39.9 / 60.1
1CsHAP	480.6 $\pm$ 59.46	20.0	69.4 / 30.6
10CnHAP	343.8 $\pm$ 35.67	36.4	59.4 / 40.6
10CsHAP	343.8 $\pm$ 35.30	20.8	44.4 / 55.6

n/a refers to not applicable as the substance does not have the material and/or was received from Pulse Therapeutics.

TABLE III

CRYSTALLITE SIZE OF HAP AND MAGNETITE IN NANOCOMPOSITE

Nanoparticle System	HAP Crystallite Size (nm)			Magnetite Crystallite size (nm)	
	(002)	(211)	(310)	(311)	(400)
ION	n/a	n/a	n/a	19.60	22.60
MB	n/a	n/a	n/a	n/a	n/a
nHAP	35.04	7.50	15.36	n/a	n/a
sHAP	30.95	7.71	20.84	n/a	n/a
1CnHAP	45.21	6.96	19.97	*	*
1CsHAP	31.76	7.26	22.46	*	41.80
10CnHAP	31.49	6.45	19.51	30.82	*
10CsHAP	31.02	7.05	21.35	29.44	*

n/a refers to not applicable as the substance does not have the material and/or was received from Pulse Therapeutics. * refers to indeterminate as the substance should have the material but the machine was not able to measure

Zeta potential measurements for ION and MB, and for nHAP and sHAP were similar, as seen in figure 7A and 7B, respectively. ION and MB have similar isoelectric points as in previous literature (52). The two points of zero charge can be best explained by the composition of the ION and MB. The material in both IONs is biphasic, containing magnetite and maghemite, giving the double hump character (53). When the pH is greater than 8, zeta potential is predominantly maghemite. When the pH is less than 8, zeta potential is predominantly magnetite. In maghemite, the concentration of oxygens is greater than magnetite (54). The oxygen groups on the surface transform to hydroxyls, their negative charge will be higher and the point of zero charge would be lower than magnetite.

HAP dominated the zeta potential curves of both 10C-HAPs at pH greater than 6. At pH lower than 6, ION dominated the zeta potential curves of both 10C-HAPs. The zeta potential curves of the 10C-HAPs can be explained by the degradation of HAP at low pH. As the pH decreases, the rate of dissolution of HAP into its component pieces increases, and in the case of HAP-ION systems, more ION is present on the nanocomposite. This means that as HAP in the HAP-ION degrades, more ION character is present, shifting the zeta potential to be predominantly that of ION.

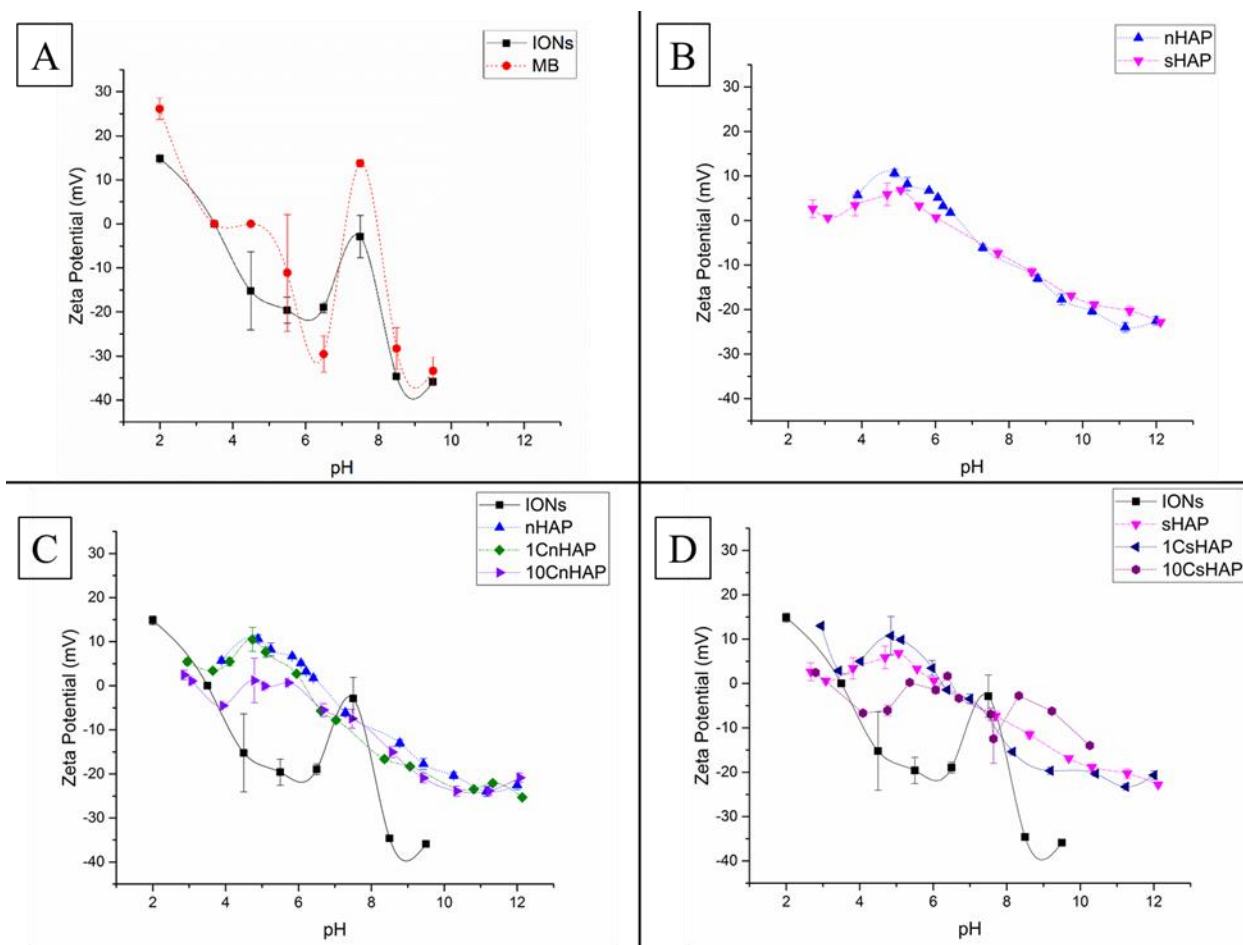


Figure 7. Zeta potential measurements of nanoparticle systems. A) ION and MB. B) nHAP and sHAP. C) ION, nHAP, 1CnHAP, and 10CnHAP. D) ION, sHAP, 1CsHAP, and 10CsHAP.

C. Magnetic Hysteresis of HAP-IONs

1CsHAP and 10CsHAP were chosen for magnetometry due to their ability to increase cell viability in MSC and decrease cell viability in the glioblastoma cell lines. As seen in figure 8, both 1C- and 10CsHAP have superparamagnetic qualities. As seen in the insert of figure 8, 1C- and 10CsHAP do have some remanence (0.048 emu/g and 0.928 emu/g, respectively) and coercivity (31.5 Oe and 24.9 Oe, respectively) not equal to zero. The magnetic saturation of 1C- and

10CsHAP are 1.08 emu/g and 15.03 emu/g, respectively. 1CsHAP has approximately 10 times less ION than 10CsHAP and the values for their magnetic saturation appear to differ to their ION concentration.

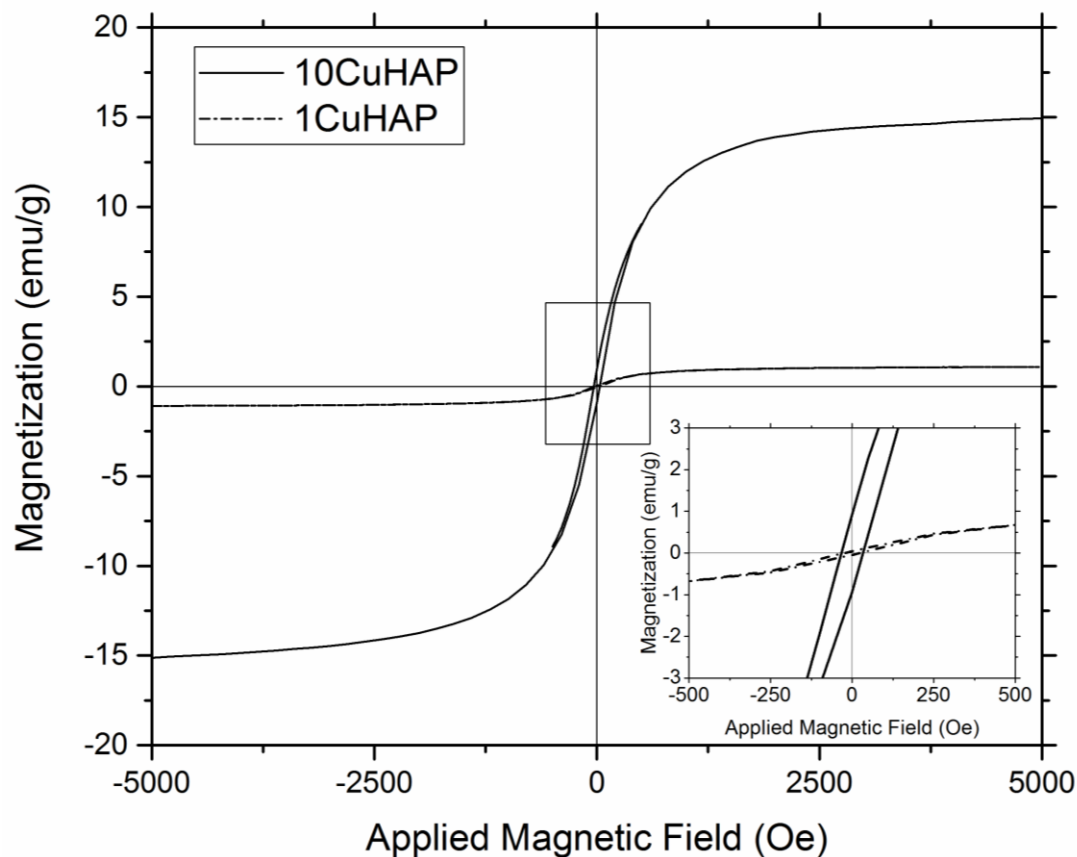


Figure 8. Hysteresis curve of 1CsHAP and 10CsHAP.

D. Nanoparticle Cytotoxicity

The HAP-IONs did not reduce the viability of hMSCs similar to the experimental control; however, at the same time they reduced the viability of both cancer cell lines. As seen in figure 9,

IONs and MB hinder cell viability in MSCs versus the experimental controls. In both cases, the lower concentration of nanoparticles harms more cells. In the case of 10CsHAP, MSCs had an increase in viability versus the negative control ($P = 0.439$). The cytotoxicity of the HAP-IONs follow the hypothesis that HAP-IONs would minimize the risk to healthy cells. It is evident in figure 9, that HAP-IONs and HAP do not alter the cell viability versus the sample control (5% water in cell media).

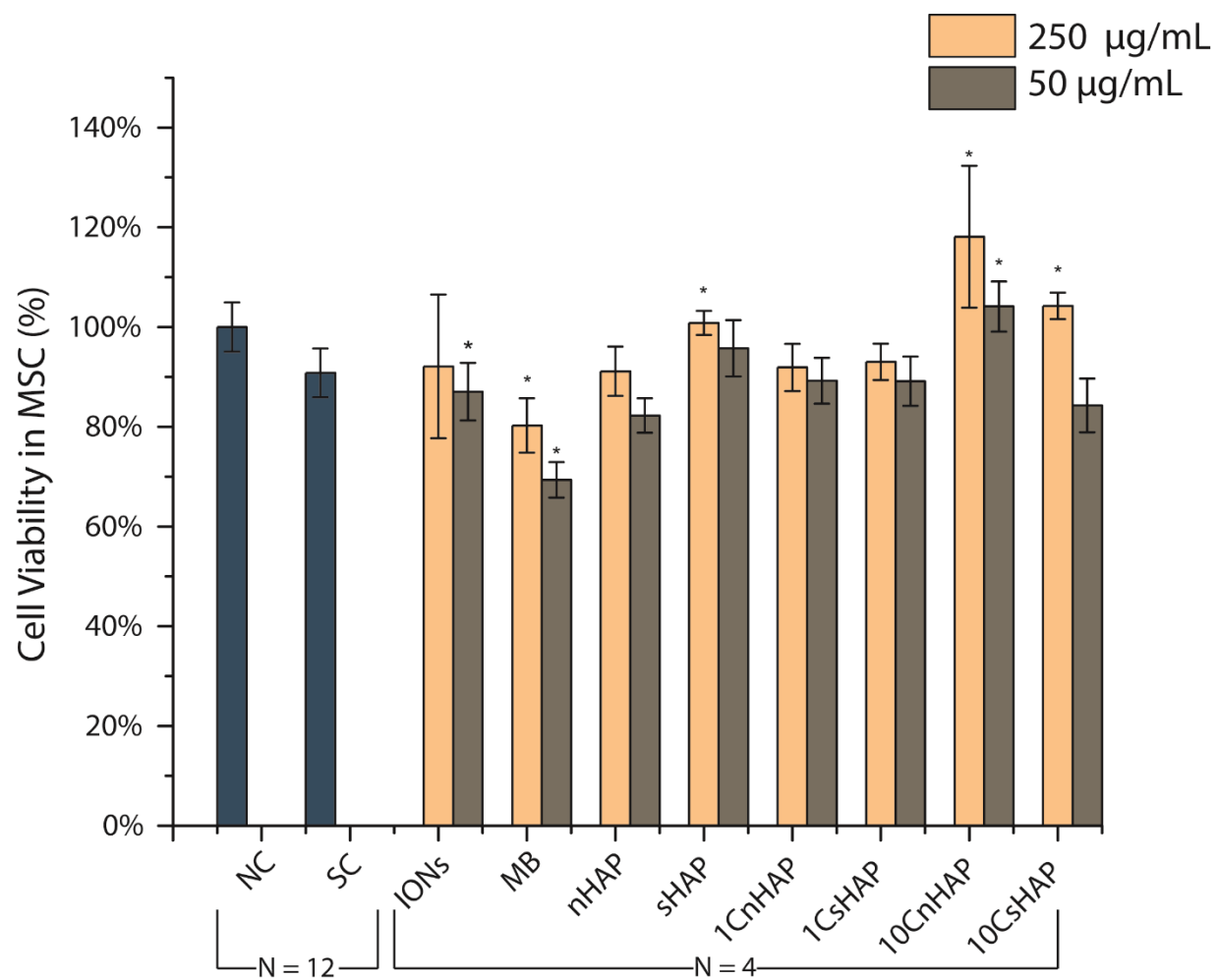


Figure 9. Cytotoxicity in human mesenchymal stem cells (hMSC) compared to IONs, MBs, HAP (nHAP and sHAP), and HAP-ION nanocomposites (1CnHAP, 1CuHAP, 10CnHAP, 10CuHAP). NC refers to negative control; SC refers to sample control; * refers to $P < 0.05$ vs SC

The nanoparticles strongly reduced the cell viability in the U87 human glioblastoma cell line, as seen in figure 10. The HAP-IONs were able to produce a greater cytotoxic effect at lower concentrations than ION and MB. Both nHAP and sHAP alone were able to reduce the viability of U87 cells by at least 35%, with nHAP at 50 $\mu\text{g/mL}$ the most cytotoxic. 1CnHAP induced the largest decrease in cell viability among the HAP-IONs, leaving only 57.80% ($P = 3\text{E-}9$) and 56.72% ($P = 7\text{E-}10$) at 250 and 50 $\mu\text{g/mL}$, respectively.

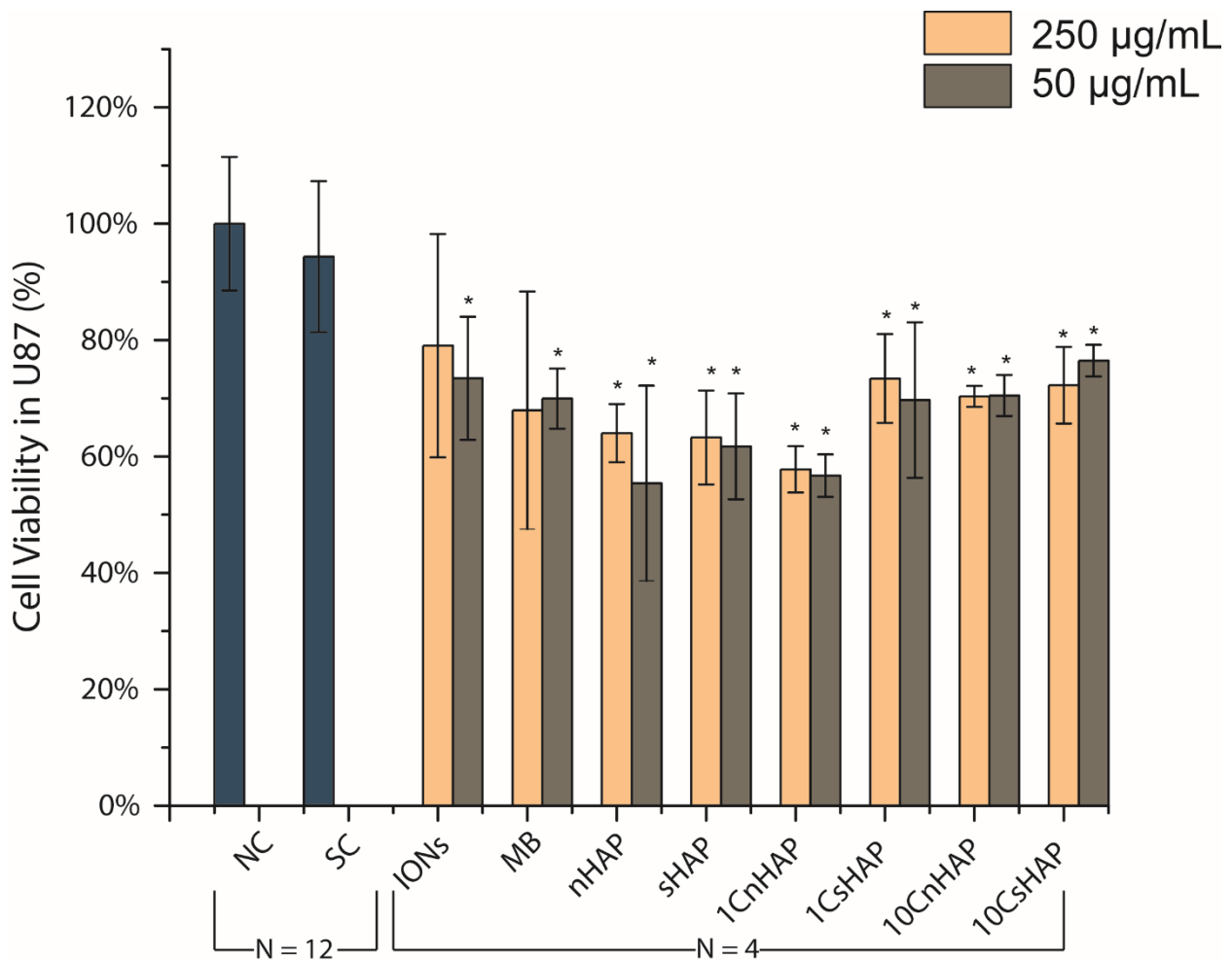


Figure 10. Cytotoxicity in U87 human glioblastoma compared to IONs, MBs, HAP (nHAP and sHAP), and HAP-ION nanocomposites (1CnHAP, 1CuHAP, 10CnHAP, 10CuHAP). NC refers to negative control; SC refers to sample control; * refers to $P < 0.05$ vs SC

The primary patient glioblastoma cell line E297 had the most peculiar responses to the nanoparticle systems. IONs did not appear to affect the viability of E297 cells. Cell treated with 250 $\mu\text{g/mL}$ had 92.11% ($P = 0.37$) viability, and cells treated with 50 $\mu\text{g/mL}$ had 87.04% ($P = 0.01$) viable for 250 and 50 $\mu\text{g/mL}$, respectively. HAP and HAP-IONs affected the E297 cells to a similar degree as U87, diminishing viability more than or equal to 16.66%, as seen in figure 11. 1CnHAP caused more cell death in E297 than in U87, leaving only 51.51% ($P = 7\text{E-}10$) and 44.87% ($P = 7\text{E-}6$) viable at 250 and 50 $\mu\text{g/mL}$, respectively.

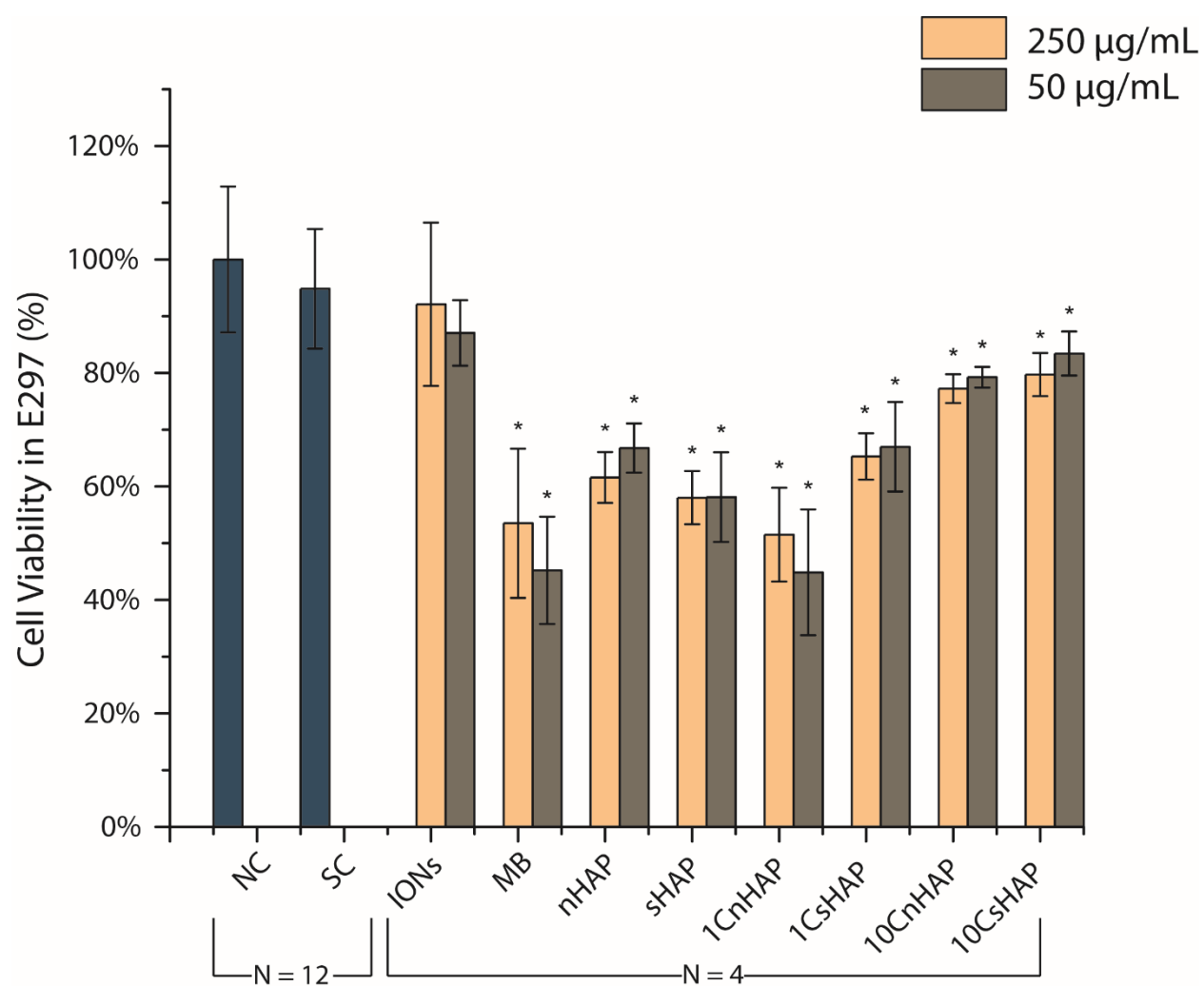


Figure 11. Cytotoxicity in E297 primary patient glioblastoma compared to IONs, MBs, HAP (nHAP and sHAP), and HAP-ION nanocomposites (1CnHAP, 1CuHAP, 10CnHAP, 10CuHAP). NC refers to negative control; SC refers to sample control; SC refers to sample control; * refers to $P < 0.05$.

E. Migration of E297 Spheroids

Migration of E297 spheroids demonstrated that the materials presented have acted as a barrier for cancer cell migration, as seen in figure 12. The control spheroids had an average migration of 478.5 μm , while the majority of the materials did not allow for more than 100 μm migration. The nanoparticle systems behaved as a physical barrier in most cases as seen in figure 13 and 14. In figure 13A, the control spheroid has long arms extending from the spheroid, but treatment of ION or MB, blocks this extension, as seen in figure 13B and 13D, respectively. Figure 13C demonstrates a change in the spheroid shape. This change was noticeable due to the increase in light passing through the sample. The change in shape resembles that of a toroid and it appears that there is less cell density in the middle of the spheroid.

In figure 14, the HAP and HAP-IONs behave like barriers as well. Due to the agglomeration of the HAP and HAP-ION particles, large crystal-like structures appear near the spheroids. Even if there is an opening for migration, as seen in figure 14A and 14C, it is difficult for the tumor spheroids to migrate due to the cytotoxicity imparted by the material.

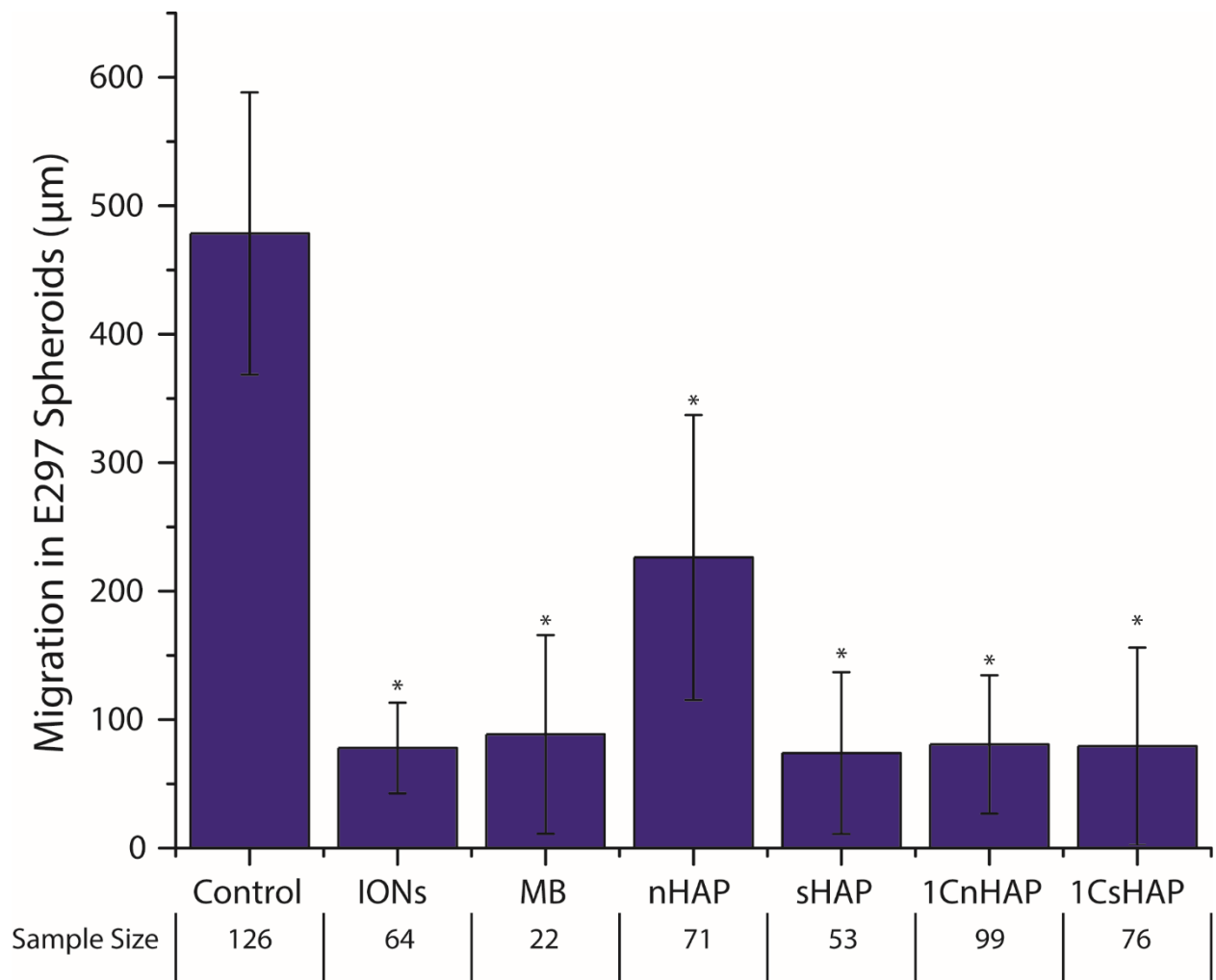


Figure 12. Average migration of E297 spheroids. * refers to $P < 0.05$. Table insert defines the number of measurements with columns as sample groups.

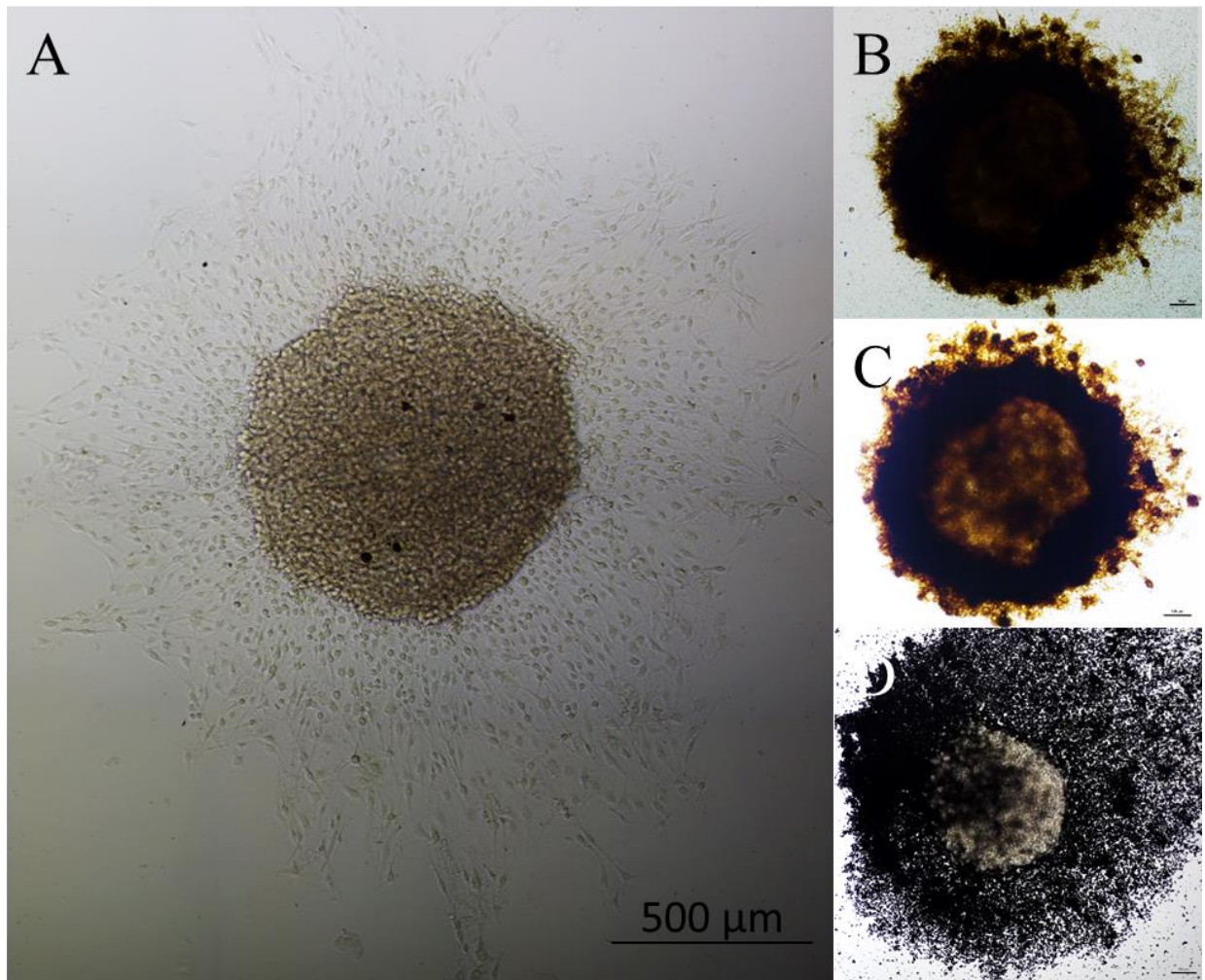


Figure 13. E297 spheroid migrations in different treatments. A) Control. B) ION. C) ION (same as B) with increased light through the sample. D) MB. Scale bars for: A) 500 μm ; B-D) 100 μm

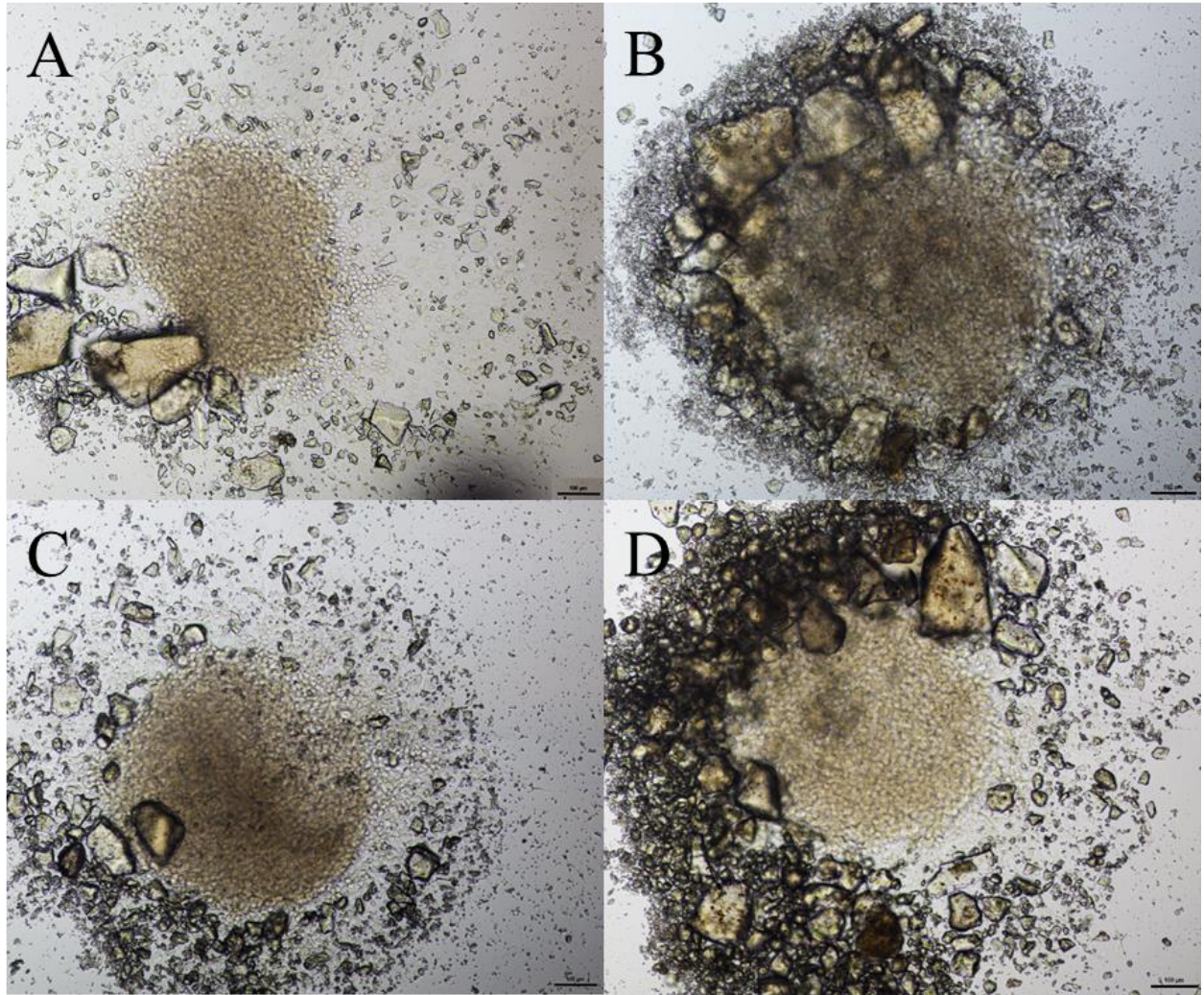


Figure 14. E297 spheroid migrations in different treatments. A) nHAP. B) sHAP. C) 1CnHAP. D) 1CsHAP. Scale bars for A-D: 100 μm .

F. Uptake of Nanoparticles

The uptake of nanoparticles in the cell lines can be seen in figure 15. As mentioned before, IONs were uptaken more in the healthy cells (MSCs) than in the cancer cells. MSC and U87 each had an increase in uptake versus ION but not E297. MSC ingested more 1CsHAP and 10CsHAP than ION ($P = 0.039$ and $P = 0.040$, respectively). U87 ingested more 1CnHAP and 1CsHAP than ION ($P = 0.0008$ and $P = 6E-7$, respectively). Only E297 did not ingest more of a nanoparticle system than ION alone. Each nanoparticle system in MSC, U87, and E297 can be seen in figure 16, 17, and 18, respectively. As seen in figures 17H, 17I, 18B, 18C, 18F, and 18G, the nanoparticles and nanocomposites have a perinuclear localization. This means that these nanocomposites are uptaken by some endocytic pathway (55).

The use of MB is to determine the effects of commercially available SPIONs against the present synthesized IONs. With the current evidence, MBs are not effectively uptake into cells. MBs litter the cell and surround areas but uptake is very poor. Without sufficient uptake, MBs would not be an effective MH device.

As seen in figure 16B-C, 17B-C, and 18B-C, ION and MB are visible solely by the red and blue channels as seen in figure 16 but not in figures 17-18. The ION and MB can be seen in the green channels of these images as seen in figure 19. ION and MB acted similarly to contrast agents under the green channel, being black under the green filter and saturating the rest of the image with green color. This property was exploited to determine the uptake of ION and MB in MSC and U87.

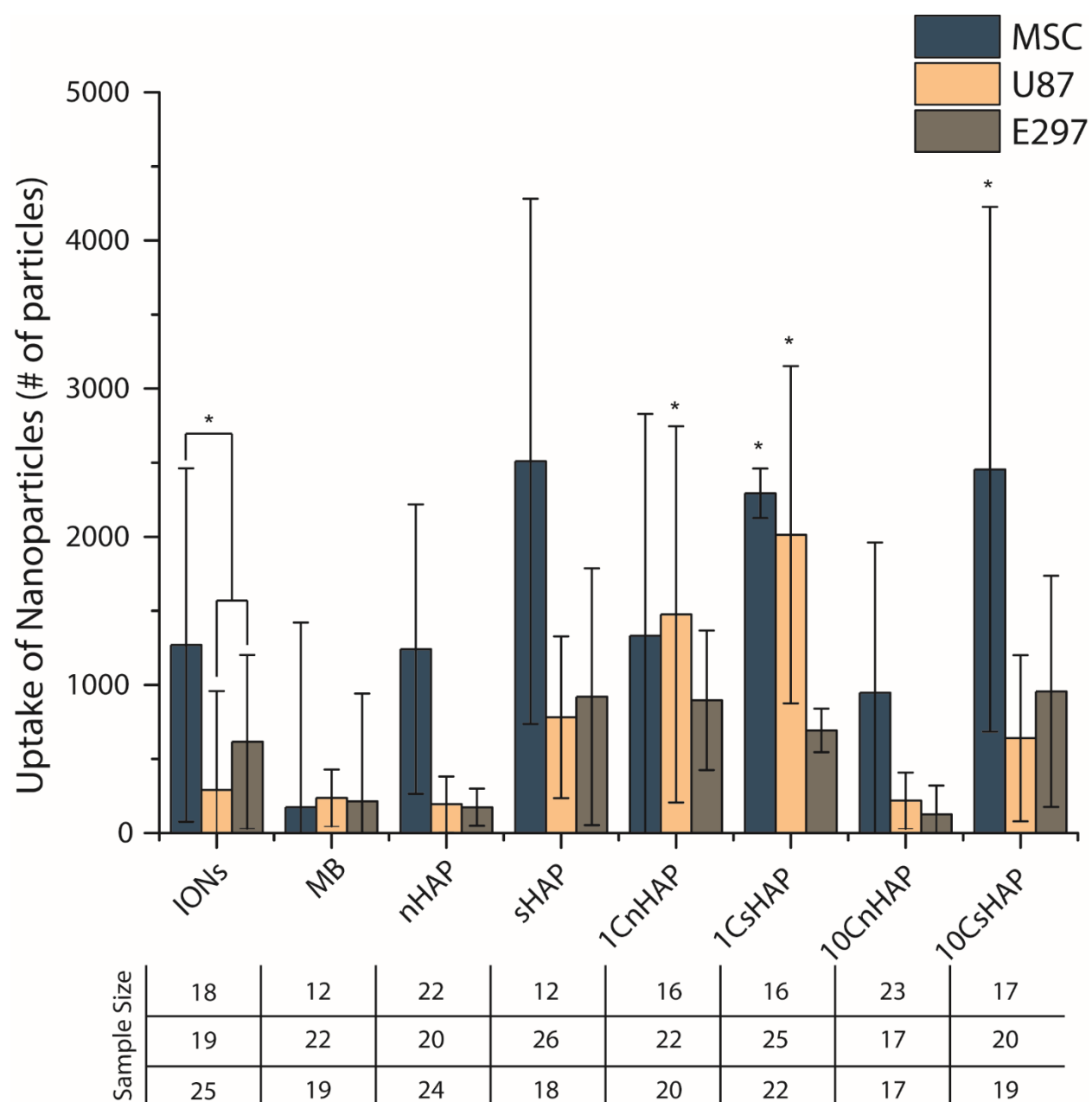


Figure 15. Uptake of nanoparticle systems in various cell lines. * refers to $P < 0.05$ of sample vs ION uptake in their cell line. Table insert defines the sample size with columns as sample groups and row as cell lines. Top row is MSC, middle row is U87, and the bottom row is E297.

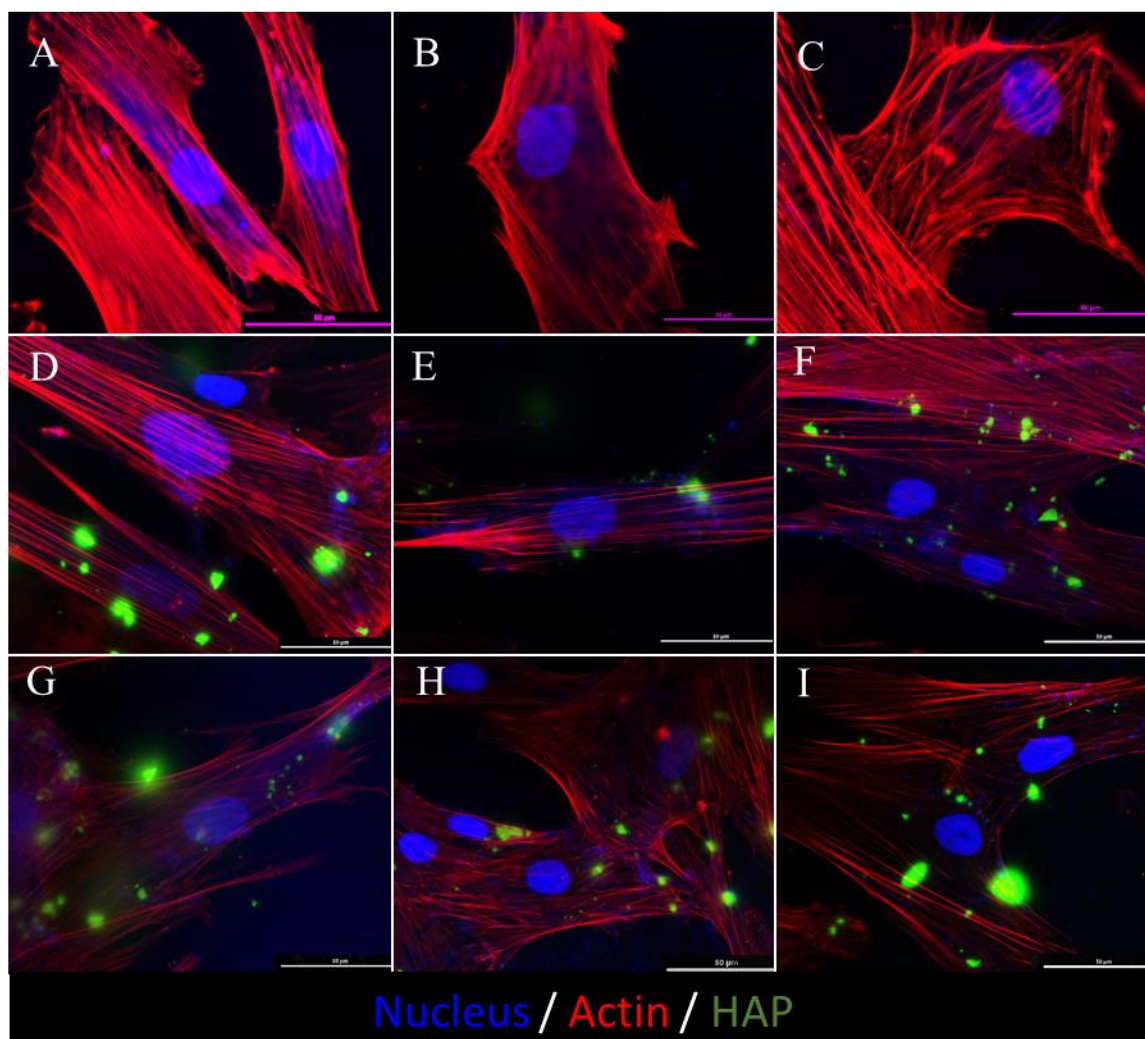


Figure 16. Fluorescence images of MSC treated with various nanoparticle systems. A) Control. B) ION. C) MB. D) nHAP. E) sHAP. F) 1CnHAP. G) 1CsHAP. H) 10CnHAP. I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm .

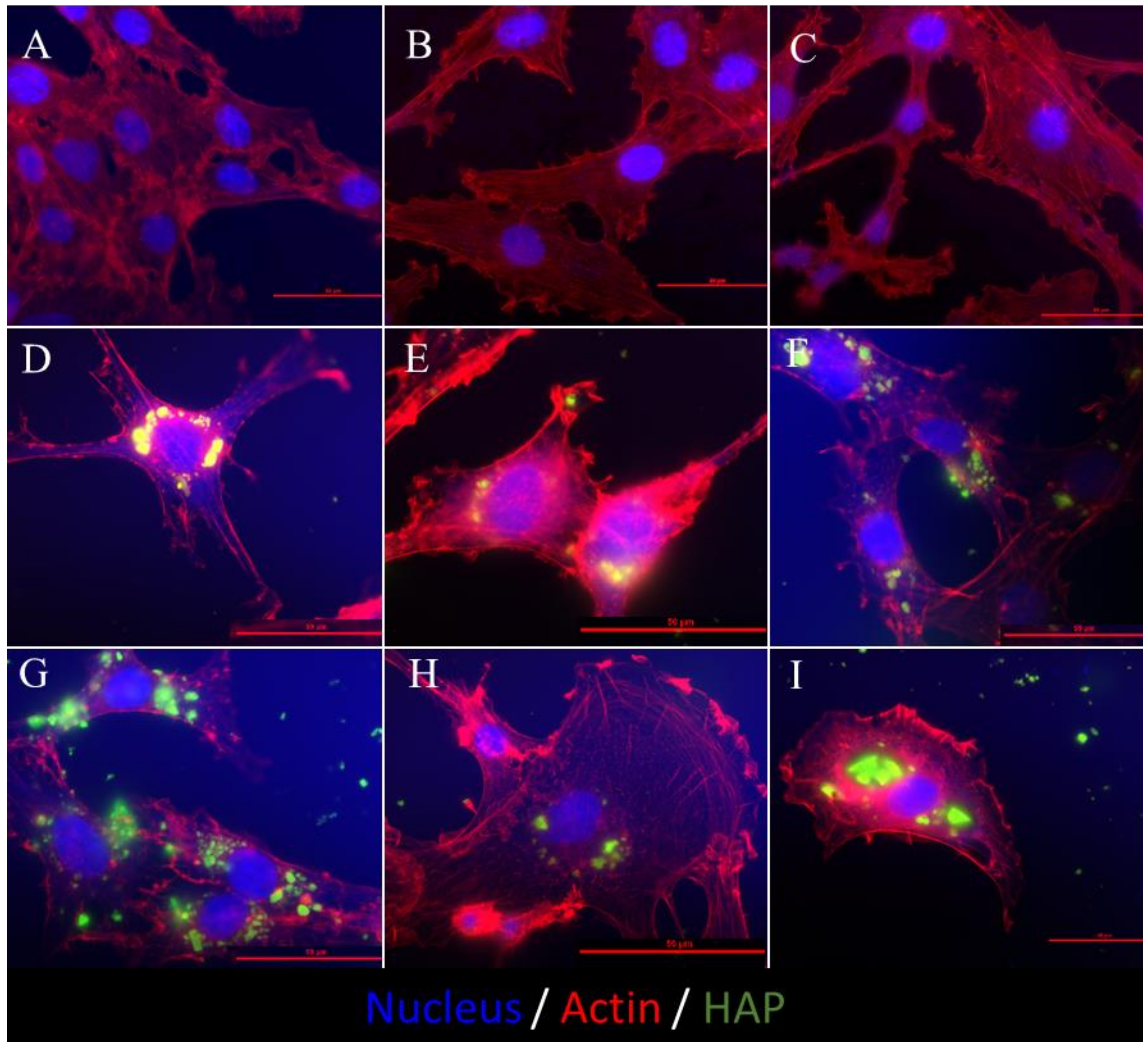


Figure 17. Fluorescence images of U87 treated with various nanoparticle systems. A) Control. B) ION. C) MB. D) nHAP. E) sHAP. F) 1CnHAP. G) 1CsHAP. H) 10CnHAP. I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm.

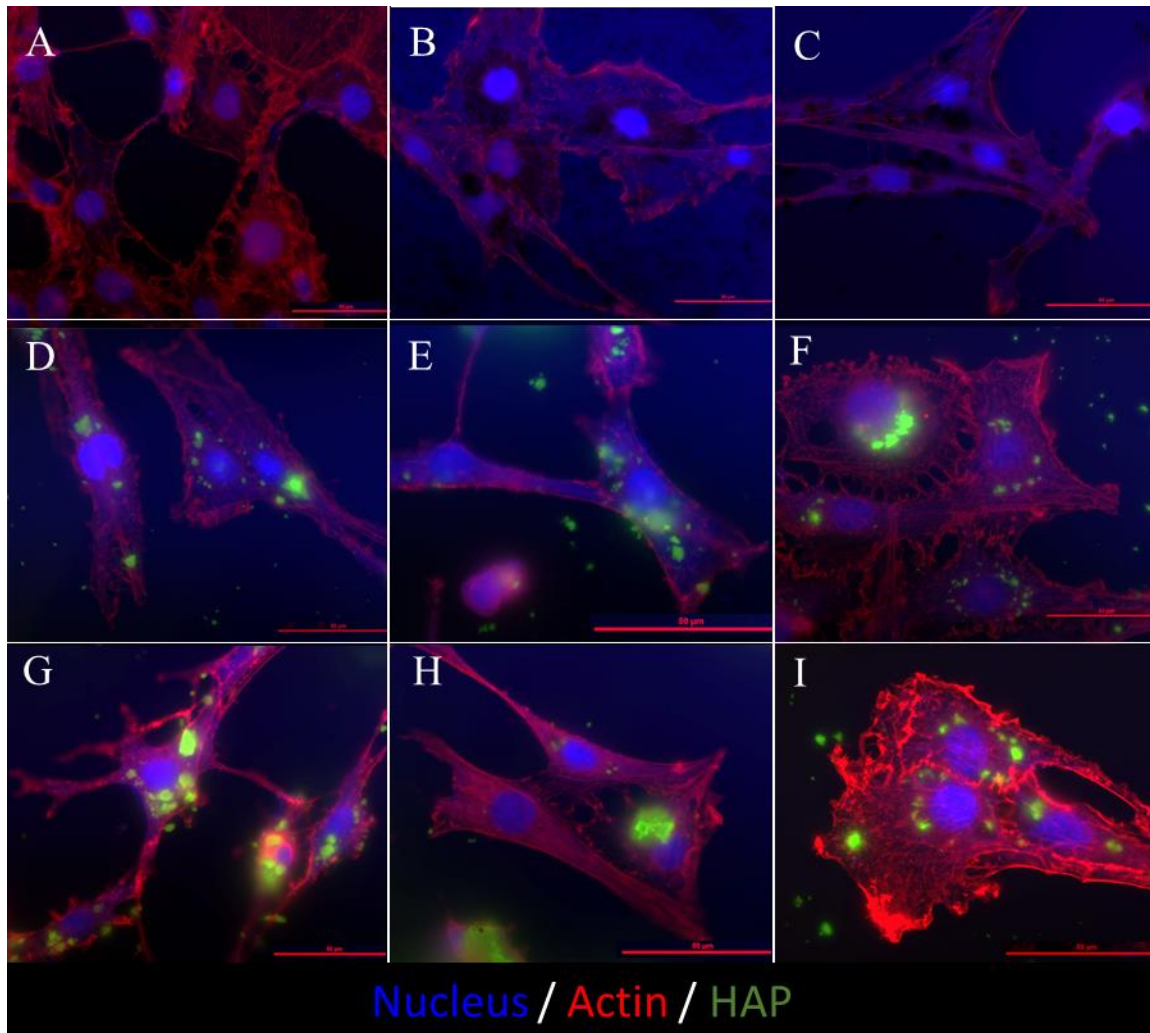


Figure 18. Fluorescence images of E297 treated with various nanoparticle systems. A) Control. B) ION. C) MB. D) nHAP. E) sHAP. F) 1CnHAP. G) 1CsHAP. H) 10CnHAP. I) 10CsHAP. The nucleus is stained blue, actin is stained red, and HAP is stained green. Scale bars are 50 μm .

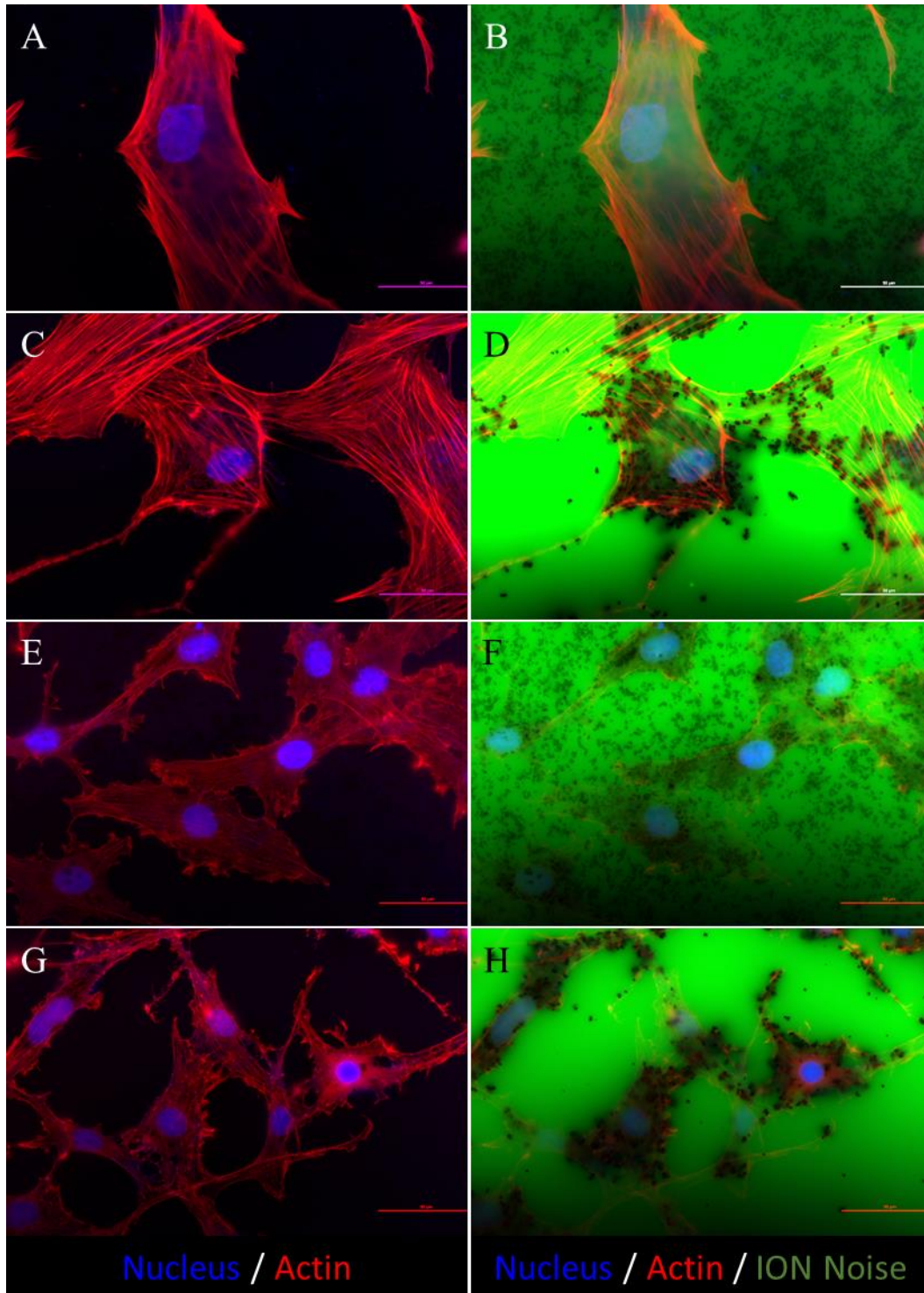


Figure 19. Fluorescence images of: (A, B, C, D) MSC; and (E, F, G, H) U87. (A, B, E, F) are ION; and (C, D, G, H) are MB. (A, C, E, G) contain red and blue channels; and (B, D, F, H) contain red, blue and green channels.

G. Change in the Anisotropy of the Actin Cytoskeleton

Utilizing FibrilTool, evaluating the changes in the actin cytoskeleton were possible. MSCs had a significant change in their cytoskeleton anisotropy during the uptake of IONs, 1CnHAP, and 10CsHAP, as seen in figure 20. ION treatment decreased the anisotropy, creating a more disorganized cytoskeleton by 0.1552, or 15.52% ($P = 0.02$). In contrast, 1CnHAP and 10CsHAP treatment increased the anisotropy, forming a more organized cytoskeleton by 0.2338 ($P = 0.0001$) and 0.1675 ($P = 0.006$), respectively.

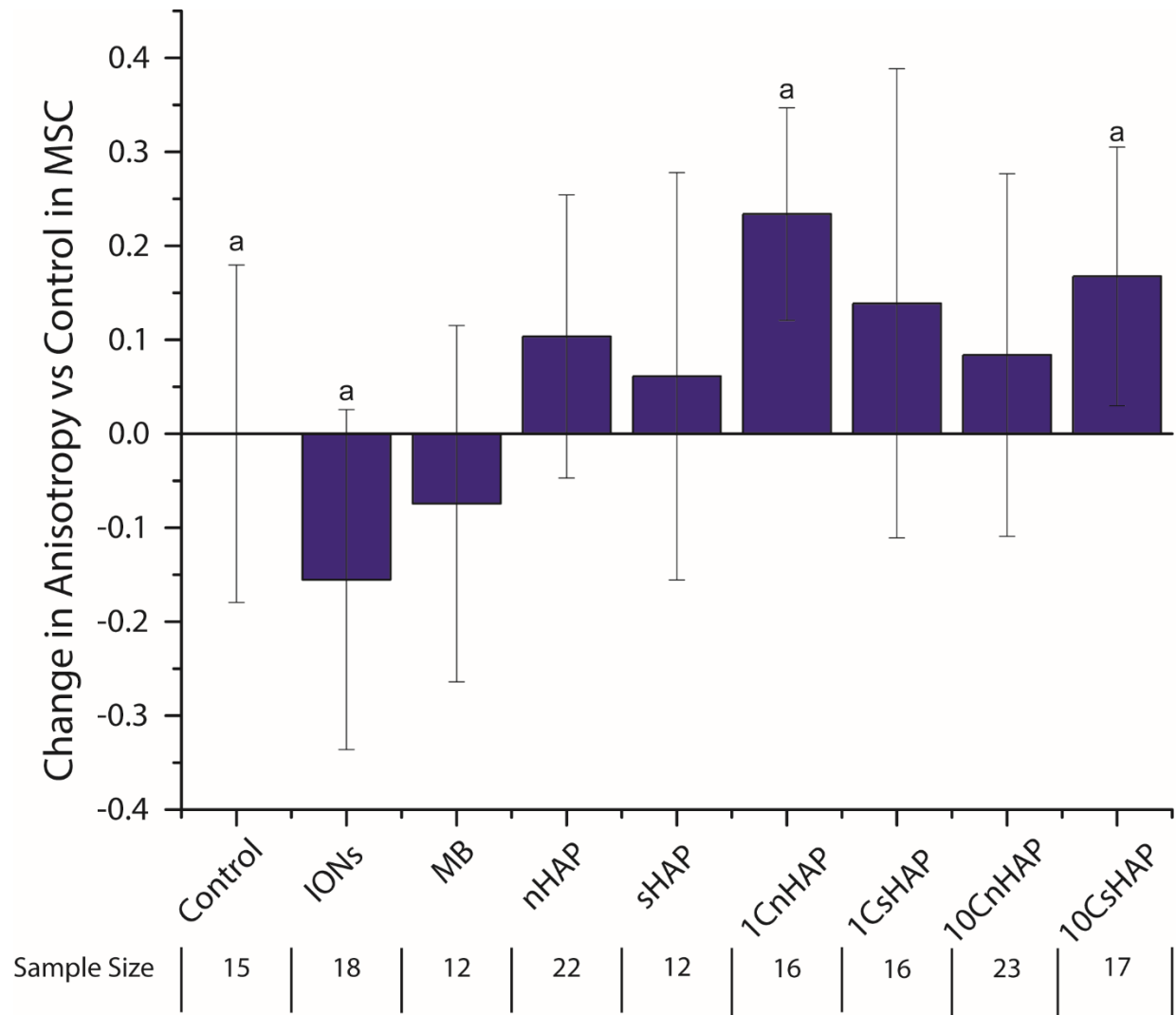


Figure 20. Change in the actin cytoskeleton anisotropy versus control in MSC. * refers to $P < 0.05$ versus control. Table insert defines the number of measurements with columns as sample groups.

In U87 cells, there was no significant changes in the actin cytoskeleton, as seen in figure 21. U87 cells had the least amount of average change versus the control in all treatment groups, barely surpassing 5%.

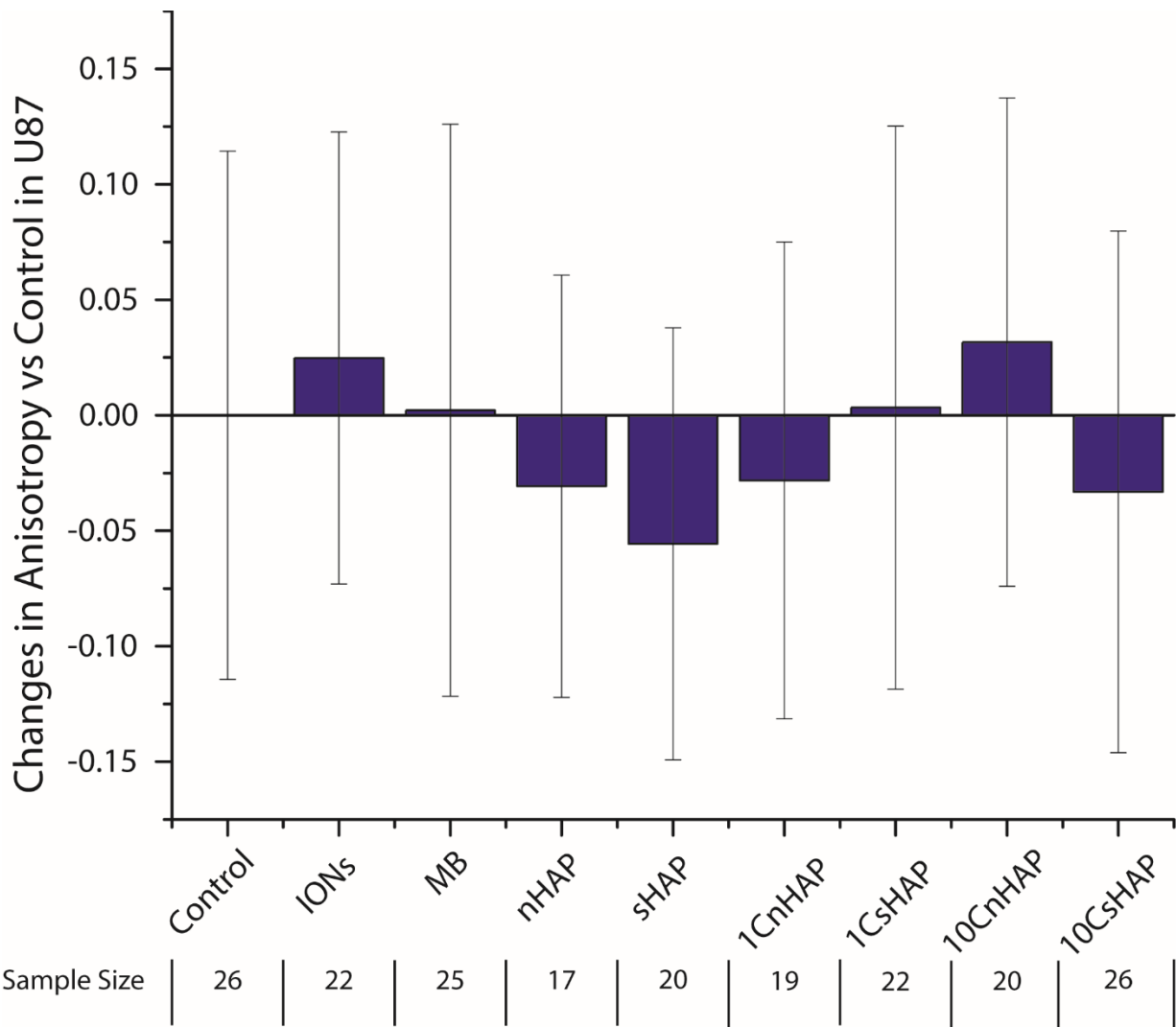


Figure 21. Change in the actin cytoskeleton anisotropy versus control in U87. Table insert defines the number of measurements with columns as sample groups.

In E297 cells, there was significant changes in the actin cytoskeleton in all samples, as seen in figure 22. E297 cells had the most amount of average change versus the control, greater than 10% and nearly surpassing 30%. The increase in anisotropy defines that the actin cytoskeleton became more organized after treatment. sHAP and MB had the first and second greatest changes in the cytoskeleton versus control, with an increase of 0.314 ($P = 1E-8$) and 0.265 ($P = 4E-6$), respectively.

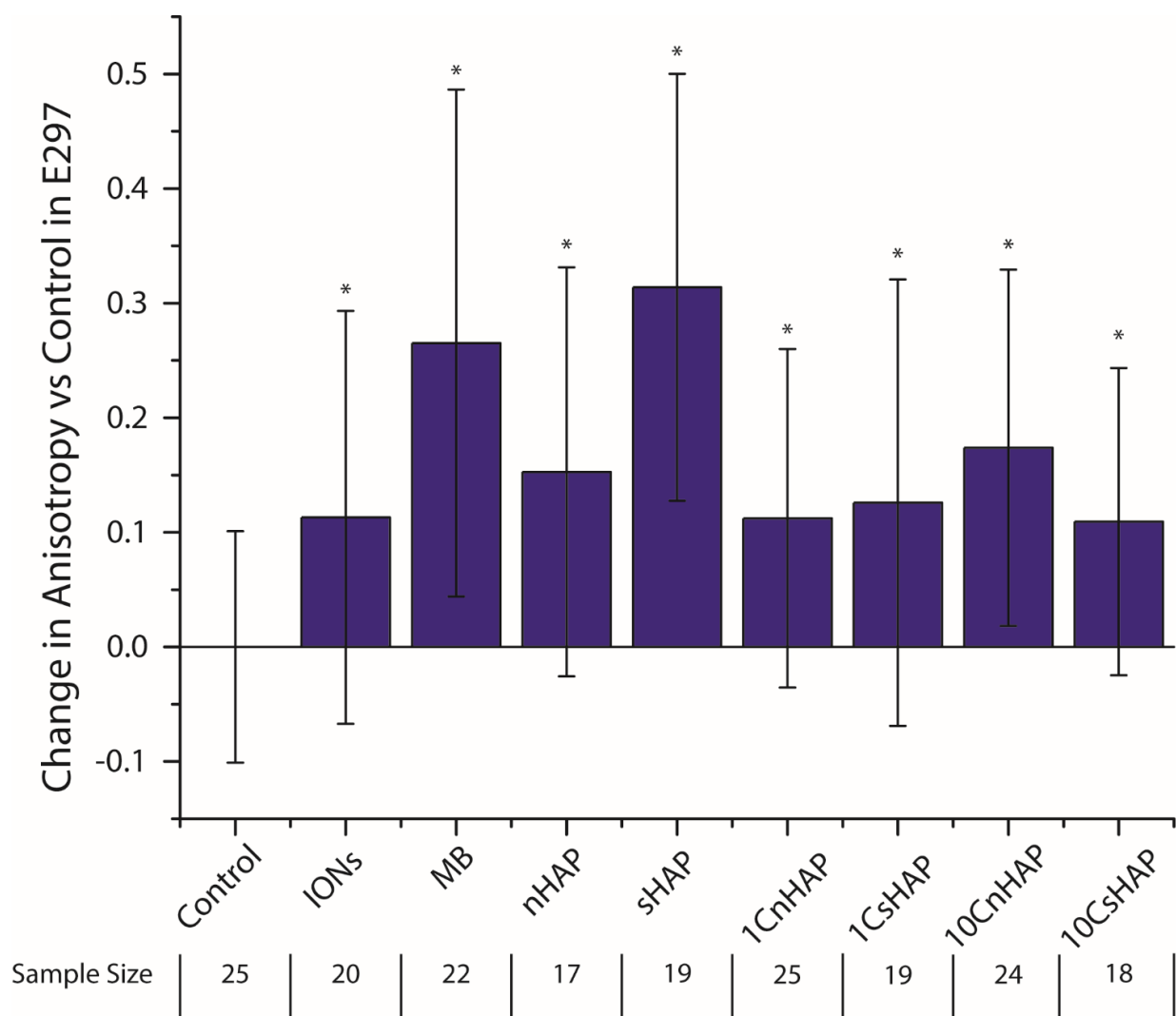


Figure 22. Change in the actin cytoskeleton anisotropy versus control in E297. * refers to $P < 0.05$ versus control. Table insert defines the number of measurements with columns as sample groups.

IV. DISCUSSION

HAP-IONs open a new avenue to novel cancer treatment therapies that can utilize MH. HAP-IONs have a superparamagnetic nature making MH possible. These particles maintain healthy cell viability and do not decrease their viability past the sample control, and have increased uptake in cancer cells. In addition, HAP-IONs reduce cancer cell viability, allowing for treatment of tumors without the need for MH, and demonstrate a slowed cancer tumor migration. Further analysis of the actin cytoskeleton demonstrates that MSC have a reduced anisotropy and a less organized actin cytoskeleton when treated with IONs, providing elementary evidence to the increased uptake of IONs in healthy cells. E297 cancer cells have an increased anisotropy and increased order when treated with HAP-IONs that correlates to the decreased spheroid migration, which adds to the elementary evidence of changes in the actin cytoskeleton anisotropy and their effects in cells. HAP-IONs can offer a new cancer treatment therapy that does not require harsh chemotherapeutics and minimizes the risk to healthy cells.

However, agglomeration and aggregation impede the full capabilities of HAP-IONs. It is evident from the DLS measurements that the HAP-IONs are non-nanosized materials, but SEM and TEM demonstrate agglomerated nanosized material. Agglomerated particles and unstable colloids reduce the efficacy of vehicle delivery to the cells (56). At physiological pH, HAP-IONs have a zeta potential of less than -15 mV. A low zeta potential between -15 and 15 mV is more likely to cause the particles to agglomerate and become unusable in treatment (57). The low zeta potential decreases the stability of any colloid including HAP (58). The agglomeration may occur before the HAP-IONs can reach the cells, and could affect the possible cytotoxicity imparted onto cells (31). For instance, the 250 $\mu\text{g/mL}$ of HAP-ION should impart greater than or equal to the cytotoxicity as HAP or ION at the same concentration. Instead, the cytotoxicity of HAP-IONs in

U87 or E297 is less than HAP or ION at the same concentration. The 50 $\mu\text{g/mL}$ of HAP-IONs appeared to impart a greater hindrance to cell viability. Logically, large concentrations of a substance should harm more cells, or at least to the same degree as HAP or ION alone. It is likely that at higher concentrations it is easier for the HAP-IONs to agglomerate creating a larger structure. This larger structure can become ineffective for MH because HAP-IONs do not enter the cell.

The current rate of agglomeration may affect the thermal conductivity of the ION. Hong *et al.* demonstrated that as iron nanofluid cluster size increases, the thermal conductivity decreases (59). This may appear to place IONs at a disadvantage, but the clustering can be reversed. Espinosa *et al.* demonstrated that applying a low power laser of 0.3 W/cm^2 , the maximum temperature reached during MH was greater than MH or laser alone (60). The iron nanocubes used by Espinosa *et al.* were clustered together within late endosomes, and the laser caused a local plasmonic heating freeing the nanocubes and restoring Brownian motion. The restored Brownian motion could then be used in MH. With this in mind, the rate agglomeration of the HAP-IONs needs to be determined as a function of the particle concentration before the conditions for the optimal uptake of HAP-IONs can be derived.

HAP-IONs retain their superparamagnetic ability making them ideal candidates for MH. Forming a nanocomposite with IONs runs the risk of losing or decreasing the magnetization of ION. Silica coated magnetite experience a decreased maximum magnetization (61) as well as polymer coatings of SPIONs experience a decreased maximum magnetization (62). With HAP-IONs, the magnetization increases nearly 15-fold when the ION concentration increased by 10-fold (from 1CsHAP to 10CsHAP). A less than 10-fold increase is expected. Maghemite has a smaller maximum magnetization (63) and is more present in 10CsHAP. The 10CsHAP

magnetization should have a less than 10-fold increase than 1CsHAP because 10CsHAP contains more of an inherently weaker magnetic material. Further experimentation is required to determine why 10CsHAP had a greater magnetization with an inherently weaker magnetic material.

IONs alone would not have been an ideal candidate for MH. IONs were uptaken more in healthy cells than in cancerous cells with the consequence being that during MH greater toxicity and necrosis would occur in healthy tissue than cancerous tissue. This work demonstrates that in hMSCs uptake of IONs occurs in a greater quantity than glioblastoma cells. Previous work demonstrated a similar pattern of high uptake of ION in healthy cells and a smaller uptake of ION in cancerous cells (64) demonstrating a common problem with ION uptake. Commercially available SPIONs should be a better candidate for MH than IONs alone, as they are formulated to minimize toxicity in healthy cells. However, MBs are less effectively uptaken into cancerous cells than IONs. The characteristics of MB are likely due to its functionalization. MBs have a short PEG coating to increase its hydrophylicity (65), and appears to affect the uptake of the particles. In all three cell lines, the standard deviations for MB uptake are greater than the average uptake even after removing outliers. MBs are highly magnetic but without their entrance into a cell, MH would be difficult to achieve because of the decreased uptake. Addition of HAP to the IONs increases uptake in cancerous cells versus ION or MB alone. U87 and MSCs uptake 1CsHAP significantly more than ION. 1CnHAP is uptaken by U87 significantly more than ION. 1C-HAPs are more spherical than 10C-HAPs. More spherical particles are easier to uptake (66) and why 10C-HAPs are not uptaken as much as their 1C-HAP counterparts. Against IONs or MBs, HAP-IONs are uptaken more into cancerous cells and become better candidates for MH.

The migration of ION treated spheroids raises the question of the effects of tumor volume and metastasis. The spheroid treated with IONs assumes a near toroidal shape and loss of tissue

mass, as seen by the increased illumination through the center of the tumor. Why does the spheroid change shape if the greatest concentration of ION is on the periphery of the spheroid? It couldn't be from the ION cytotoxicity alone, because its cytotoxicity was not statistically significant against E297 experimental controls. When ION was added into the well containing a spheroid, a small portion of the ION concentration covered the top of the spheroid because the spheroid was submerged in media. The spheroid treatments lasted 72 hours, and as time passed, the IONs would begin to settle on the bottom of the well, where cells would migrate out from the spheroid. One of two things is possible. Either, cells in the center or cells in the middle are more prone to cell death; or there is a change in intercellular communication. Cells in the center of a tumor are more prone to necrosis because diffusion of material deep into a tumor is difficult without angiogenesis (67), but intercellular communication can be altered changes in exosomes and vesicular carriers (68) or by altered cell migration (69). The uptake of IONs was less than that of hMSCs but ION treatment significantly increases the anisotropy of actin cytoskeleton in E297. It is likely that uptake of nanomaterials caused cytoskeletal changes, and perhaps tilted cells towards cell death that affected the shape of the spheroid. ION treatment appears to either halt or greatly decrease the migration of cells out of the spheroid, though the possibility exists that due to the cytotoxicity of the IONs, the lack of cell migration could be from increased cell cytotoxicity of the migrating cell population because of the increased concentration of settled nanoparticles on the periphery of the spheroid.

Similarly, HAP-IONs halted and/or decreased cell migration from the spheroid, though there was no apparent changes in contrast of the spheroid center. 1CnHAP had a smaller average cell migration than its nHAP counterparts, while 1CsHAP had a similar average cell migration than its sHAP counterparts. With the previous evidence about uptake, 1CnHAP becomes the best

candidate for MH, offering greater uptake, reducing cell migration, and reducing cytotoxicity in healthy, primary cells.

In this work, preliminary evidence on actin cytoskeletal rearrangement after nanoparticle uptake is presented. The actin cytoskeleton becomes more disorganized in hMSC decreases with an increased ION uptake. As actin cytoskeleton organization increases in hMSC, we see a decreased HAP-ION uptake versus the uptake of the individual materials HAP or ION. For instance, hMSC cells treated with 1CnHAP had a statistically significant increase actin cytoskeleton organization and had an uptake of 1CnHAP less than that of ION or nHAP alone.

E297 spheroid migration supplements that fact; decreased cell migration from the spheroid occurs due to increased cytoskeletal rearrangement. E297 cells experiences an increase in cytoskeletal organization after HAP-ION uptake. E297 spheroids treated with HAP-IONs had very little migration. Increased organization in the actin cytoskeleton correlates with diminished spheroid migration. Experimentation with U87 spheroids is necessary to determine if glioblastomas other than a patient derived glioblastoma experience similar migration and anisotropy patterns because U87 is an established cell line dating back to 1968 (70). E297 is a patient cell line extracted in 1991 (71) that does not require immunosuppressants to grow tumors on rats (72). It is likely that E297 cells exhibit a more malignant tumorigenicity than U87 cells.

It is known that in certain cases the actin cytoskeleton determines the extent of the uptake of a material. One hypothesis is that nanocomposite uptake affects macropinocytosis. Macropinocytosis is an actin-dependent endocytic pathway (73,74) and actin cytoskeletal rearrangement may affect macropinocytosis. However, the cytoskeletal rearrangement seen here could be an indirect effect of nanoparticle uptake. The reorganization could be due to cytotoxicity of the particles inducing cell death or decreasing cell health and the actin rearrangement is a

downstream product of the effects of the nanoparticles on cell health (75). Further experimentation is necessary to determine if macropinocytosis is affected by actin cytoskeletal rearrangement. In addition, further work will be required to determine what the exact mechanism of action of the nanoparticles is in cancer cells.

However before MH can become a viable clinical cancer treatment, HAP-IONs need to be tested with MH *in vitro* and *in vivo*. Previous studies with HAP-IONs have constructed MH systems and tested the heating efficiency without cells (46,76). The previous studies demonstrate that HAP-IONs can increase temperature of the surrounding area but do not test the effect in cells. HAP-IONs have been introduced into *in situ* tumors in mice and shown to greatly reduce the volume of the tumor after MH treatment (77). There have been clinical trials with patients presenting glioblastoma multiforme but only one group in Europe has been able to achieve positive results using MH and IONs (18). In the current treatment setup, MH will have to be applied concurrently with other cancer therapy treatments until nanoparticles can weaken a cell with MH and effectively decrease cancer cell viability, such as HAP-IONs.

In conclusion, HAP-IONs provide a new nanocomposite cancer treatment device that utilizes biocompatible material to decrease cancer cell viability and maintain healthy cell viability. These nanocomposites slow cancer spheroid migration and retain their superparamagnetic ability. Cumulatively, HAP-IONs provide a new MH device for cancer therapy that does not require harsh chemotherapeutics and minimizes the risk to healthy cells.

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VI. APPENDICES

A. MATLAB CODE

1. Localization.m

```
%% Localization of Nanoparticles - Sebastian Pernal - 16 May 2016

clear, clc, close all

% Load image

numNPs = zeros(25,1);

for i=1:25

    A = uigetfile('.tif');

    A = imread(A);

    Ar = A(:,:,1); % Red part

    Ab = A(:,:,3); % Blue part

    Ag = A(:,:,2);

    Abr = Ab-Ar; % Just the blue

    Abrg = im2bw(Abr, 0.15);

    areaAbrg = bwarea(Abrg); % Total number of pixels

    factor = 0.23; % micrometers per pixel

    number = factor*areaAbrg; % total micrometers in the image

    sizeNP = 150/1000; % 150nm in micrometers

    numNPs(i) = number/(150/1000);

end
```

2. LocalizationBlackfromRB.m

%% Localization of Nanoparticles - Sebastian Pernal - 16 May 2016

clear, clc, close all

% Load image

% numNPs = zeros(25,1);

nImages = 26;

number = zeros(nImages,1);

for i=1:nImages

 A = uigetfile('.tif');

 A = imread(A);

 Ar = A(:, :, 1); % Red part

 Ab = A(:, :, 2); % Green part

 Ag = A(:, :, 3); % Blue part

 Ag = imcomplement(Ag);

 Abr = Ag-Ar; % Just the green

% Abr = imcomplement(Abr);

 Abrg = im2bw(Abr, 0.9);

 areaAbrg = bwarea(Abrg); % Total number of pixels

 factor = 0.05; % micrometers per pixel

 number(i) = factor*areaAbrg; % total micrometers in the image

% sizeNP = 150/1000; % 150nm in micrometers

% numNPs(1) = number/sizeNP;

end

3. LocalizationBlackfromGreen.m

%% Localization of Nanoparticles - Sebastian Pernal - 16 May 2016

clear, clc, close all

% Load image

img = 16;

number = zeros(img,1);

for i=1:img

% i=1;

A = uigetfile('.tif');

A = imread(A);

Ar = A(:,:,1); % Red part

Ab = A(:,:,2); % Green part

Ab = imcomplement(Ab);

Ag = A(:,:,3); % Blue part

% Abr = Ab-Ag; % Just the green

% Arb = Ar-Ab;

% Abr = Abr-Arb;

Ag = im2bw(Ag, 0.5);

Ab1 = im2bw(Ab, 0.8);

Abr = Ab1-Ag;

```

Abrg = im2bw(Abr, 0.1);

areaAbrg = bwarea(Abrg); % Total number of pixels

factor = 0.05; % micrometers per pixel

number(i) = factor*areaAbrg; % total micrometers in the image

end

```

4. **LocalizationGreen.m**

%% Localization of Nanoparticles - Sebastian Pernal - 16 May 2016

```
clear, clc, close all
```

```
% Load image
```

```
nImages = 18;
```

```
number = zeros(nImages,1);
```

```
for i=1:nImages
```

```
% i=1;
```

```
    A = uigetfile('.tif');
```

```
    A = imread(A);
```

```
    Ar = A(:, :, 1); % Red part
```

```
    Ab = A(:, :, 2); % Green part
```

```
    Ag = A(:, :, 3); % Blue part
```

```
    Ag = im2bw(Ag, 0.5);
```

```
    Ab = im2bw(Ab, 0.1);
```

```
    Abr = Ab-Ag; % Just the green
```

```
    Abrg = im2bw(Abr, 0.10);
```

```
    areaAbrg = bwarea(Abrg); % Total number of pixels

```

```
factor = 0.05; % micrometers per pixel  
number(i) = factor*areaAbrig; % total micrometers in the image  
end
```

VII. VITA

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Pernal, Sebastian. "Photoacoustic Imaging of Biological Tissues." *UBSJ* (2014): 12