RICE UNIVERSITY

Nanomaterials for X-ray imaging and image-guided therapy applications

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TEXAS
December 2016
ABSTRACT

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In recent years, nanomaterials have been explored for a wide range of different applications. Their tunable size and composition allow them to cross cellular membranes, and therefore, various nanomaterials have been used for a diversity of medical applications such as drug, gene and protein delivery, as well as for diagnostic tools in the form of imaging agents. This work presents the study of two new carbon nanotube (CNT)-based materials as radiocontrast agents for stem cell labeling and imaging. X-ray-based imaging modalities are the most used and readily available diagnostic imaging tools in the clinic today; however, this technology is not the conventional method for imaging and tracking of stem cells. Hence, this work introduces the use of CNT materials as contrast agents (CAs) for X-ray computed tomography (CT) imaging, as an alternative to magnetic resonance imaging (MRI), which is currently the technology of choice for this purpose.

The first material described in this thesis, termed Bi@US-tubes, consists of ultra-short CNTs (US-tubes, 20-80 nm), loaded with bismuth ions. Bi@US-tubes contain 2-3% bismuth by weight and its use as an intracellular CA for mesenchymal stem cells (MSCs) has been studied. As a second generation material, Bi₄C@US-tubes has also been prepared, characterized, and used to intracellularly label MSCs. Bi₄C@US-tubes are derived from US-tubes and Bi(III)-oxo salicylate clusters which localize at defect sites and
along the sidewalls of the US-tubes. $\text{Bi}_4\text{C}@\text{US}$-tubes contain 20% bismuth by weight, and therefore, this new material exhibits higher X-ray attenuation than $\text{Bi}@\text{US}$-tubes. The labeled MSCs showed high viability regardless of whether $\text{Bi}@\text{-US}$-tubes or $\text{Bi}_4\text{C}@\text{US}$-tubes were used as the intracellular CA. The proliferation, differentiation, and other properties of the labeled MSCs were also studied, which showed no major differences when compared to control cells.

The final part of this thesis describes the preparation and characterization of a new formulation containing an anticancer drug, heparin, and an iron oxide nanoparticle, all of which are FDA-approved compounds. The three components interact to one another by electrostatic interactions to form a nanocarrier that can be manipulated and targeted by strong external magnetic fields. The release of the drug, doxorubicin, from the nanocarrier was found to be pH-dependent, with faster release occurring under acidic conditions. This new formulation has the potential to be used in the clinic as an image-guided therapy when using magnetic targeting in conjunction with MRI tracking.
Acknowledgments

Research is best accomplished and is most enjoyable when bright and wonderful people are part of the process. This thesis could never have come to completion without the contribution of many great individuals who made this journey possible. I will try to enumerate those who in one way or another helped me throughout this adventure, and my apologies to those who I may have forgotten to mention. First, I would like to thank those who were involved with the work itself, starting with my advisor Prof. Lon J. Wilson, for giving me the opportunity to join his group and for providing me with guidance and support for the past five years. Prof. Wilson also gave me the opportunity to be involved in numerous projects beside the ones described in this thesis, as well as in review articles, all of which I appreciate very much. I also greatly appreciate Prof. Angel A. Martí, Prof. Jane Grande-Allen and Dr. Maria da Graça Cabreira for taking the time to serve on my thesis committee and for revising my work.

The “Wilsonites” have become an extended family and many of them have contributed significantly to this work. My former undergraduate research assistant, Stephen Y. Cho participated significantly in the work described on Chapters 3, 4 and 5. Stephen spent many long hours digesting samples with strong acids, preparing ICP samples, and making the bismuth-carbon nanotube materials, an essential and time consuming labor, of which I am very grateful. Besides being of great help in the laboratory, Stephen was a tremendous friend and lab member. Big thanks to Dr. Lesa Tran for the training during my first year. Dr. Tran served as my mentor (and good friend!) at the beginning of my graduated education, giving me support, constant encouragement, and extensive scientific
advice that have stayed with me throughout these years. Words cannot fully describe the role that Dr. Yuri Mackeyev plays in Prof. Wilson’s lab. I would like to thanks Dr. Mackeyev specially for preparing innumerous batches of chloric acid, for keeping every instrument in the lab running, for the help with the THF and AFM computer, helpful scientific advice, and for obtaining the HPLC data presented in Chapter 6. Dr. Richa Sethi, Dr. Meghan Jebb and Dr. Sophia Phounsavath (last name which I never learned how to pronounce correctly) for training me in different instruments, helpful insights and scientific discussions, cutting of carbon nanotubes (and training), and most importantly for such amazing friendships, many laughs and long lunch getaways. Dr. Matthew Cheney for training me on the AFM, as well as for being my run partner on the not-so-hot days in Houston. Dr. Ayrat Gizzatov for the many interesting conversations during “tea time”, helpful discussion, and for giving me the opportunity to contribute to one of his projects. Dr. Ish Kumar, as well as Prof. Kenton H. Whitmire for contributing to the work described in Chapter 4. Dr. Sakineh E. Moghaddam for the HR-TEM images presented in Chapter 4. A big thanks to Nicholas Zaibaq for obtaining NMR data, helpful discussion, endless conversations, and for being my desk neighbor and lunch buddy for the last two years. I would like to thank Dr. Nadia C. Lara, Dr. Stuart Corr, Dr. Adem Guven, Dr. Eladio Rivera, Dr. Maciej Serda, Dr. Justin J. Law, Tyler Buchaman, Michael Collins, Brandon Cisneros, and Ari Berlin for all their help and company during my time in the Wilson group.

I would like to express my deep appreciation to Dr. Maria da Graça Cabreira and Dr. Emerson C. Perin for serving as my second advisors at the Texas Heart Institute. The work presented in Chapters 3 and 5 were done fully in collaboration with them. Graça, thank you for being such a wonderful friend and advisor, and for all the support that you
have giving me from my very first year. Thank you to the entire family at the Texas Heart Institute and the Baylor St. Luke’s Medical Center for making me feel like one of their own. Special thanks go to Dr. Xiaohong Wang, Alon R. Azares, Dr. Ralph Nichols, Dr. Maximillian Buja, Pamela Potts, Dr. Amy Caivano, Dr. Raja Muthupillai, Tracye Dauphin, and Dr. James Willerson. To Dr. Benjamin Y. Cheong for obtaining the CT images presented in Chapters 4 and 5. Special thanks to Dr. Stephen Curly and his group at Baylor College of Medicine for the helpful discussions, and Dr. Merlyn X. Pulikkathara for the STEM images. This work was supported by the Robert A. Welch Foundation (Grant C-0627, LJW).

Words cannot express how thankful I am to family and friends for keeping me sane, happy and motivated during the past five years. To my parents and siblings, thank you for all your love and support and for always encouraging me to pursue my dreams. You guys have been my biggest cheerleaders, thank you! To my dearest friends at home, Paula, Aime and Zenaida. To Cynthia, Janice and Karla, for staying along my side throughout college and beyond. To my Houston family, Tomi, Sara, Francesca, Gladys, Martina, and many others, for making me feel at home. To Adriana for being my workout partner, confident and dearest friend. To Charya, for being the best roommate and friend that someone can ask for. To Geoffrey, for being my rock during the last year. Thank you for all the patient, love, support, and encouragement that you have given me, for that and more I am eternally grateful.

This dissertation is dedicated to Mom, Dad, Wildaliz, Gerardo, Sebastián, & Zahir.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>αMEM</td>
<td>Alpha-minimal essential medium</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Bi@US-tubes</td>
<td>US-tubes containing Bi$^{3+}$ ions</td>
</tr>
<tr>
<td>Bi$_4$C@US-tubes</td>
<td>US-tubes containing Bi(III)-oxo salicylate clusters</td>
</tr>
<tr>
<td>CA(s)</td>
<td>Contrast agent(s)</td>
</tr>
<tr>
<td>CBS</td>
<td>Citrate-buffered saline</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony-forming unit-fibroblast</td>
</tr>
<tr>
<td>CNT(s)</td>
<td>Carbon nanotube(s)</td>
</tr>
<tr>
<td>CT</td>
<td>X-ray computed tomography</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DOX-H</td>
<td>Complex containing doxorubicin and heparin</td>
</tr>
<tr>
<td>DOX-HFe</td>
<td>Complex containing doxorubicin, heparin and ferumoxitol</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy dispersive spectroscopy</td>
</tr>
<tr>
<td>EE %</td>
<td>Encapsulation efficiency percent</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>GNT(s)</td>
<td>Gadonanotube(s)</td>
</tr>
<tr>
<td>HR-TEM</td>
<td>High resolution-transmission electron microscopy</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively-coupled plasma-mass spectrometry</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively-coupled plasma-optical emission spectrometry</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenium</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance Imaging</td>
</tr>
<tr>
<td>MSC(s)</td>
<td>Mesenchymal stem cell(s)</td>
</tr>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
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<td>Population doubling time</td>
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<td>Scanning electron microscopy</td>
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Chapter 1

Introduction

1.1. Overview

The use of stem cells for disease treatment and regenerative medicine has exploded in recent years as an encouraging new medical field. In stem cell-based therapies, the poor engraftment and survival of stem cells represent two major problems, challenging the effectiveness of these therapies. Therefore, it is essential to be able to track stem cells in vivo to better understand the outcome of stem cell therapies. This has motivated researchers to develop a wide range of in vitro cell labeling techniques. Tracking of cells allows scientists to monitor stem cell activity under physiological conditions, their retention rate at target sites, their biodistribution, and their eventual clearance from the body. One of the easiest and most used techniques to track stem cells involves the intracellular labeling of the cells with an imaging agent that is active in one or multiple imaging technologies. Nanoparticles, including carbon nanotubes (CNTs), have been successfully used for such applications.
The hydrophobic nature of CNTs allow them to cross cell membranes without the need of transfection agents, making this nanomaterial suitable to transport associated materials into the inner space of cells for therapy, imaging, or combined applications. In this thesis, the study of two new CNT-based materials as intracellular contrast agents (CAs) for mesenchymal stem cell (MSC) labeling is presented. Both of these nanomaterials, Bi@US-tubes and Bi₄C@US-tubes contain bismuth ions in the CNT platform. These new agents have demonstrated attenuation of X-rays due to the high concentration of bismuth with them, and thus, they can serve as CAs for X-ray-based technologies, such as radiography, X-ray computed tomography (CT), micro computed tomography (micro-CT) and fluoroscopy. The potential of these materials when internalized in MSCs, as well as the behavior of the MSCs after being exposed to the agents for prolonged time, have been evaluated and are discussed in the following chapters.

Furthermore, this thesis also presents the preparation and study of a magnetic nanoparticle coated with a polymeric layer which contains an anticancer drug. Short and long term side effects caused by chemotherapy are some of main concerns of cancer therapy and many strategies to reduce toxicity are currently under study. In this regard, the targeting of anticancer drugs using strong magnetic fields is one of the main approaches that has been extensively studied. This is possible when the anticancer agent is associated to magnetic nanoparticles, such as superparamagnetic iron oxide nanoparticles. Furthermore, the release of the drug from the magnetic nanocarrier is of great importance to determine the effectiveness of the treatment, doses needed, and how often the drug should be administrated. The formulation described in this thesis contains Feraheme®, heparin, and
doxorubicin, all FDA-approved materials, which makes this new composite material a powerful tool that can be used for pre-clinical and clinical trials within the next few years.

1.2. Organization

This dissertation is divided into five main chapters. Chapter 2 discusses the background literature, as well as the motivation for the present work. It presents, not just the work that have been reported previously, but also the science and principles behind some of the materials and techniques discussed in the following chapters. Chapter 3 presents the characteristic of porcine MSCs after being labeled with Bi@US-tubes. Chapter 4 presents the synthesis and full characterization of the Bi₄C@US-tube material as a new second generation radiocontrast agent. In Chapter 5, the use of Bi₄C@US-tubes as an intracellular CA for MSCs is presented, and the results are compared to those obtained for the Bi@US-tube material. Finally, Chapter 6 aims to present the preparation and characterization of an easy-to-make nanocomplex containing doxorubicin and two other FDA-approved drugs, including a magnetic nanoparticle. The new formulation can potentially be used as a magnetic nanocarrier for the drug doxorubicin, to increase selectivity and reduce drug side effects. The conclusion and future directions for each project can be found at the end of each chapter.
2.1. Carbon nanotube-based radiocontrast agents for stem cell tracking

2.1.1. Mesenchymal stem cells (MSCs)

Stem cells possess great potential for different medical applications and every year more investigators join this field of study. As interest in stem cells has increased, it has become essential to track the cells in vivo in order to study their biodistribution and possible tissue accumulation. One of the main sources of adult stem cells is the bone marrow, where a number of different stem cell populations are found, including hematopoietic stem cells.

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1 Part of this chapter has been published in the following review article: M. Hernández-Rivera, N. G. Zaibaq, L. J. Wilson, Toward carbon nanotube-based imaging agents for the clinic. Biomaterials, 2016, 101, 229-240.
(HSCs) and mesenchymal stem cells (MSCs). Of these, MSCs have the advantage of being less immunogenic than other stem cells due to the lack of the co-stimulatory molecules of the B7 family that are required to initiate an immune response.\textsuperscript{1–3} This allows the use of MSCs without concern for immunological rejection or the need for immunosuppressant drugs, making MSCs a universal stem cell source. Furthermore, MSCs are easy to harvest and isolate from other types of cells, and also they can be easily expanded in culture.\textsuperscript{4} A MSC is a multipotent type of stromal cell (connective tissue cell), and its current definition include: plastic adherence in cell culture, specific surface antigen expression (CD105\textsuperscript{pos}, CD90\textsuperscript{pos}, CD73\textsuperscript{pos}, and CD45\textsuperscript{neg}, CD34\textsuperscript{neg}, HLA-DR\textsuperscript{neg}) and multi-lineage \textit{in vitro} differentiation potential (osteogenic, chondrogenic, and adipogenic).\textsuperscript{5} Currently, there are around 500 clinical trials involving MSCs reported,\textsuperscript{6} a number which has increased by a factor of three in the last five years.\textsuperscript{7}

2.1.2. Tracking MSCs

With the rapid increase in MSC-based therapies, there is an urgent need to track the MSCs \textit{in vivo} during preclinical and clinical studies to further understand and evaluate their behavior and biodistribution. To address this, various imaging techniques have been investigated, including X-ray CT,\textsuperscript{8} magnetic resonance imaging (MRI),\textsuperscript{9–14} optical imaging (such as bioluminescence and fluorescence),\textsuperscript{15–17} ultrasound-guided photoacoustic (US/PA) imaging,\textsuperscript{18} single-photon emission computed tomography (SPECT) imaging,\textsuperscript{19} and positron emission tomography (PET) imaging.\textsuperscript{20} Of these techniques, optical imaging has been shown to be a potent tool in preclinical small animal studies, but its use alone is not translatable to clinical practice due to its low penetration depth of light in biological
The use of each of these imaging techniques for stem cell tracking is reviewed in detail elsewhere. Currently, the most used and preferable imaging modality for stem cell tracking is MRI with $T_2/T_2^*$ contrast, acquired when stem cells are labeled with superparamagnetic agents such as iron oxide nanoparticles. In particular, MRI is a useful technique since it is a non-invasive imaging modality that does not suffer from issues such as tissue penetration depth or contrast resolution limitations. However, a significant clinical limitation of MRI is its incompatibility with various medical and life support devices such as pacemakers and defibrillators, the presence of which can lead to serious issues such as magnetic field interactions, heating and artifacts in the images. Another disadvantage is the required long scan periods (20 – 90 min), where the patient must remain still in an enclosed space which can cause claustrophobia. For these reasons, other imaging modalities are being sought and investigated as an alternative approach to MRI stem cells imaging.

Carbon nanotubes (CNTs) have been utilized as capsules or platforms to transport imaging agents to the inner space of cells in a safety manner, rendering the cells active in one or multiple imaging modalities (Figure 2.1). After labeling MSCs with CNT-based materials, the cells appear black in color due to the high concentration of CNT within the cells.
Figure 2.1 – Representation of the labeling of cells using CNTs as delivery agents to render the cells active in an imaging modality.

2.1.3. Single-walled carbon nanotubes

CNTs can be described as graphene sheets that are rolled in a cylindrical shape (Figure 2.2) and have different electrical and optical properties depending on the axis about which they are rolled, which is called chirality or the “twist” of the nanotube. The different CNT chiralities include the armchair structure, in which the C-C bonds are perpendicular to the tube axis; the zig-zag structure, in which the C-C bonds are parallel to the tube axis; and the chiral structure, in which the C-C bonds lie at an angle with respect to the tube axis.\textsuperscript{26,27} Single-walled CNTs (SWCNTs) are composed of a single graphene sheet and have a diameter around 1-2 nm. Some of the methods that are generally used to produce CNTs are arc-discharge, laser-ablation, and chemical vapor deposition, including the high-pressure carbon monoxide (HiPCO) synthesis. These processes usually occur in the presence of transition metal (Co, Fe, Ni) or rare earth (Gd, Y) catalysts, which remain in the pristine CNT product.\textsuperscript{28–34} Significant progress has been accomplished in the removal of trace amounts of these catalysts,\textsuperscript{34–38} although more work is currently underway to remove more of the metal catalysts. The type of CNT used in the work presented here has been produced by the arc-discharge method, with Y and Ni as the catalysts (Carbon Solution Inc., Riverside, CA, USA).
Since their discovery,\textsuperscript{40} CNTs have been of great interest because of their unique structural and chemical properties, such as their high tensile strength, high aspect ratio, and the capability to be chemically functionalized, while remaining relatively inert.\textsuperscript{41–44} This interest has led to CNTs being used in a variety of applications in electronics,\textsuperscript{45,46} material composites,\textsuperscript{47} energy,\textsuperscript{48} catalyst supports,\textsuperscript{49,50} and particularly those in the medical field.\textsuperscript{51–53}

### 2.1.4. SWCNTs in medicine

Within the emerging field of nanomedicine, CNTs have been investigated as drug delivery vectors,\textsuperscript{54,55} therapeutic agents exploiting microwave-, photo-, or radiofrequency-induced thermal effects,\textsuperscript{56–59} scaffolds for tissue engineering,\textsuperscript{60} and diagnostic imaging agents.\textsuperscript{61,62} For diagnostic and therapeutic applications, it is essential to determine the CNT biodistribution and pharmacokinetic profile to study the biological effects of the material on specific tissues. To this end, researchers have used a wide range of imaging modalities that rely on the intrinsic properties of CNTs. These techniques include Raman scattering,\textsuperscript{63} high optical and near infrared (NIR) absorbance, and photoluminescence,\textsuperscript{64–66} photoacoustic,\textsuperscript{67–70} thermoacoustic,\textsuperscript{71} and echogenic properties.\textsuperscript{72} However, some of these imaging techniques suffer from penetration depth limitations, poor spatial resolution, or
poor soft-tissue contrast.\textsuperscript{73,74} To combat these limitations, CNTs may be employed as a scaffold or capsule for radionuclides or ions that are used for the noninvasive, full-body penetrating clinical modalities, such as magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET), and X-ray computed tomography (X-ray CT) (Figure 2.3).

![Figure 2.3 - Schematic of the versatile CNT platform. Various ways to modify CNTs: attaching chelated ions or nanoparticles, such as superparamagnetic iron oxide nanoparticles (SPIONs), to the outer surface of the material or loading other agents or ions within the hollow interior of the material. Adapted from Ref. 75.](image)

While common imaging contrast agents (CAs) generally are not able to cross the cell membrane, the use of CNTs allows for these agents to be delivered intracellularly for cell tracking and sometimes selectively with the use of biological targeting moieties. Packaging the imaging agents onto or inside the CNTs enables them to be internalized by cells, without the need of cytotoxic transfection agents. However, the highly hydrophobic
nature of CNTs also prohibits their suspension in aqueous media, and several methods are used to make CNTs water-suspendable, to later use them in biological settings (Figure 2.4).

Figure 2.4 - Common CNT functionalization techniques. Common strategies to render CNTs water-suspendable including (A) non-covalent functionalization with surfactants that wrap the CNT according to van der Waals forces, (B) oxidation of the CNT surface using strong acids, (C) covalent functionalization of the surface, where R is usually a peptide chain or another hydrophilic moiety (Note: 1,3-dipolar cycloaddition of azomethine ylides and cyclopropanation are typically performed separately on different materials), and (D) non-covalent functionalization with amphiphilic peptides (blue) where a hydrophobic chain (red) interacts with the material by van der Waals forces. Figure reproduced with permission from Ref. 75. Copyright (2016), with permission from Elsevier.
2.1.5. Ultra-short SWCNTs (US-tubes)

For biological applications, the length of the SWCNTs is critical, and it is preferable to be homogeneous and in the nanometer range. Raw SWCNTs are in the micrometer range, and many techniques have been developed to shortened the CNTs, including the fluorination method. This method consists of exposing the SWCNTs to a constant flow of $F_2(g)$, which causes the breakage of the carbon-carbon bonds to form $\sim C_2F$ groups. The addition of fluorine occurs in a self-organizing manner, around the outer surface of CNTs in multiple, separated bonds. The second step involves the removal of the fluorine by etching of the fluorinated sites to create short nanotubes. Various methods have been used for this etching step, such as the use of hydrazine (to de-fluorinate the SWCNTs) followed by piranha solution with ammonium persulfate, or the use of high temperatures under an inert atmosphere (pyrolysis). The length of the US-tubes produced by fluorination followed by acid treatment or pyrolysis are 100 – 500 nm or 20 - 80 nm, respectively.

The US-tubes produced by Wilson and coworkers, including the US-tubes described in this work, were prepared via the fluorination/pyrolysis process (Figure 2.5), followed by other steps described in Chapter 4. This particular US-tube platform has been previously used to encapsulate Gd$^{3+}$-ion clusters and Gd$^{3+}$ chelates for magnetic resonance imaging (MRI), radioactive atoms for positron emission tomography (PET), Bi$^{3+}$-ions and I$_2$ for X-ray-based imaging, and chemotherapy drugs such as cisplatin, among others.
Figure 2.5 - Representation of the cutting process of SWCNTs via fluorination/pyrolysis. The first step consists of breaking the C-C bonds by exposing the SWCNTs to F$_2$(g), followed by etching of the fluorinated sites by pyrolysis.

2.1.6. X-ray computed tomography (CT)

Despite the introduction and growth of MR, ultrasound, and nuclear imaging systems, X-ray-based techniques remain one of the main imaging modalities used today due to their cost-effectiveness and diverse applicability. X-ray computed tomography (CT) was introduced for the first time in 1972 by the English engineer G. N. Hounsfield as the first of the modern slice-imaging modalities for clinical applications. Today, true volume imaging can be produced thanks to the introduction of spiral scanning and an image of organs or a full body can be obtained in 5 to 20 seconds with sub-millimeter isotropic resolution. When a conventional X-ray radiography is taken, a 2D image of the 3D
structure of the body is obtained. In contrast, CT combines multiple X-ray projections taken from different angles to produce detailed cross-sectional images of the body to produce a 3D reconstructed image (tomographic reconstruction).

For CT, the X-ray tube (generally with energy levels ranging between 20 and 150 keV) is placed on the opposite side from the detector, and these two move synchronically in a spiral manner around the patient (Figure 2.6). The beam emits $N$ photons (monochromatic) per unit of time and the rays pass through the tissue of thickness $\Delta x$. The detector then measures $\Delta N$ photons identifying the degree of attenuation of the matter. Contrast enhancement comes largely from the photoelectron effect due to atoms with high atomic numbers, thus hard tissue such as bone and cartilage have higher attenuation compared to soft tissues due to the high concentration of calcium. However, the difference in contrast between different soft tissues is sometimes too small, and in these cases, an X-ray CA is needed.

Figure 2.6 – Conventional X-ray radiography vs. X-ray computed tomography. Adapted from Ref. 87.
2.1.7. X-ray CT contrast agents

Radiocontrast agents, or X-ray CT contrast agents (CAs) are used to provide transient contrast enhancement in X-ray-based imaging modalities such as radiography, CT, and fluoroscopy. Contrast enhancement comes largely from the photoelectron effect due to the high atomic number of some elements and, as a rule, materials possessing high densities (ρ) and larger atomic numbers (Z) absorb X-rays better. The X-ray absorption coefficient (μ) expresses the relationship between the X-ray absorption phenomenon and atomic number

\[
\mu = \frac{\rho Z^4}{AE^3}
\]

Equation 2.1 – Formula for the X-ray absorption coefficient.

Where A is the atomic mass of the element and E is the incident X-ray energy.\textsuperscript{87,88} Because Z is raised to the fourth power, a small increase in Z will result in a significant
increase in μ. Thus, CAs containing heavier elements have a greater μ, which allows for greater X-ray attenuation. The ability of matter to attenuate X-rays is measured in Hounsfield units (HU). By definition, water has a HU value of 0 and air has a value of -1000 HU, while most soft tissues falling between 30-100 HU. The HU of a material with a linear μ is defined as\(^{87,88}\)

\[
HU = \frac{(\mu - \mu_{\text{water}})}{\mu_{\text{water}}} \times 1000
\]

**Equation 2.2 – Hounsfield units (HU) value of a material.**

Radiocontrast agents with good X-ray attenuation (high HU values) facilitate the process of distinguishing the region of interest, thus optimizing the contrast resolution of the image. Currently, there are two types of CAs approved for clinical use: barium sulfate suspensions (\(Z_{\text{Ba}} = 56\), administrated orally or with an enema) and small, water-soluble iodinated molecules (\(Z_{\text{I}} = 53\), commonly administrated intravenously (i.v.).\(^{88,89}\) However, both of these types of agents are exclusively for extracellular use. More recently, research has focused on the use of small molecules or nanoparticles with atoms of high atomic number (such as metals).\(^{89}\) For example, gold nanoparticles (\(Z_{\text{Ag}} = 79\)) are an ideal alternative since gold has both a high density and a high atomic number, and it provides about 2.7 times greater contrast per unit weight than iodine.\(^{90-94}\) Another material that has been investigated as a CT CA is tantalum oxide (\(\text{Ta}_2\text{O}_5\), \(Z_{\text{Ta}} = 73\)) nanoparticles. Again, \(\text{Ta}_2\text{O}_5\) nanoparticles possess better CT attenuation properties than iodinated agents on a per mole basis, and its use has been investigated both *in vitro* and *in vivo* studies.\(^{95-98}\) Bismuth-based CAs are considered to be one of the best alternatives of all metal-containing CT CAs.
Bismuth is one of the heaviest and densest metals \((Z_{\text{Bi}} = 83)\), which is located next to lead in the periodic table, and it does not present high levels of toxicity. For decades, bismuth has been used in cosmetic and medical formulations, and its toxicity profile has been extensively studied.\(^9\)\(^{9-106}\) In addition to long circulation times and safety profiles, materials containing bismuth, such as bismuth sulfide \((\text{Bi}_2\text{S}_3)\) nanoparticles and hybrid nanocrystals of iron oxide and bismuth oxide, have showed excellent results for both \textit{in vitro} and \textit{in vivo} CT contrast enhancement, long circulation times, and safety profiles.\(^107\)\(^{112}\)

Little research involving CNT-based CAs for X-ray imaging has been developed, mostly due to the high CA concentration needed to produce good contrast and the poor water solubility and dispersion achieved with CNTs in aqueous media. Previously, oxidized US-tubes filled with \(I_2\) were synthetized and the X-ray attenuation was tested, although no \textit{in vitro} or \textit{in vivo} studies were performed.\(^113\) More recently, \(\text{Bi}^{3+}\) ions and \(\text{Bi}_2\text{O}_3\) were encapsulated in US-tubes using \(\text{BiCl}_3\) as the bismuth source (\(\text{Bi}@\text{US-tubes}\)). The study of this material as an intracellular CA is discussed in Chapter 3.

### 2.2. Encapsulation of doxorubicin in an iron oxide-heparin complex

#### 2.2.1. Anticancer drugs – Challenges

Cancer cells often undergo rapid growth and proliferation compared to healthy cells, thus, anticancer drugs are compounds that are mostly toxic to cells that are rapidly growing and dividing.\(^114\) Anticancer drugs are usually administrated as free drug solutions (a single drug or a cocktail) by intravenous bolus or infusion. However, these cytotoxic agents are toxic not just to cancer cells, but to healthy cells as well. For example, many
large solid tumors have a slower cell growth kinetics than normal bone marrow cells, epithelial lining cells, reticuloendothelial system cells, and gonad cells,\textsuperscript{115} which make these tissues particularly susceptible to the majority of anticancer drugs in a dose-dependent manner. Besides being cytotoxic, anticancer drugs have other problems such as poor specificity and drug-induced resistance. Conventionally administered anticancer drugs indiscriminately bind to body tissues and serum protein in a highly unpredictable manner increasing systemic drug toxicity.\textsuperscript{116,117} In the last two decades, researchers have been working on developing drug carrier systems for targeted-delivery methods for anticancer drugs in an effort to reduce side effects and long-term toxicity.

Solid tumors have particular mechanical and physiological challenges when it comes to drug delivery. The density by surface area of the microvessel and capillary network of malignant tumors is higher than for benign tumors and healthy tissue.\textsuperscript{118} Thus, vascular permeability of tumor tissue is critically important for the delivery of drugs based on the enhanced permeability and retention (EPR) effect in cancer treatment.\textsuperscript{119} The EPR effect is of great importance for cancer treatment since the chaotic microenvironment in tumors makes it difficult for cytotoxic agents to penetrate the tumor. Thus new strategies to enhance drug penetration in cancerous tumors have the potential to improve therapeutic outcomes and reduce systematic toxicity.

\textbf{2.2.2. Doxorubicin}

Doxorubicin (DOX) is an anticancer drug naturally produced by the bacterium \textit{Streptomyces peucetius}, and is sold as DOXIL\textsuperscript{®} (doxorubicin HCl liposome injection) or as Adriamycin\textsuperscript{®} or Rubex\textsuperscript{®}. It is administered i.v. as a hydrochloride salt and is commonly
used in regimes along with other drugs for the treatment of many types of cancers, including carcinomas (solid tumors), tissue sarcomas, and hematological malignances (blood cancers such as leukemia and lymphoma).\textsuperscript{120} DOX has a molecular weight of 542.52 g/mol, and it has a resonance network composed of two benzene and two cyclohexene rings consecutively align next to each-other. Thanks to this network, DOX has an inherent fluorescent ($\lambda_{\text{ex}}$ = 498 /$\lambda_{\text{em}}$ = 588)\textsuperscript{121} and it can thus be observed by fluorescence imaging techniques \textit{in vivo}, such as confocal microscopy. Proposed action mechanisms for the cytotoxicity of DOX include (1) the intercalation of DOX between base pairs in the DNA helix, thereby preventing DNA replication, (2) disruption of topoisomerase-II-mediated DNA repair, and (3) generation of free radicals which produce damage to cellular membranes, DNA, and proteins which can also lead to oxidative stress which triggers apoptotic pathways.\textsuperscript{122,123} Despite the great chemotherapy potential that DOX possesses, a major limitation to its use is the well-known cardiotoxicity effect that the drug causes.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{chemical_structure.png}
\caption{Chemical structure of doxorubicin (DOX).}
\end{figure}

The mechanism for the cardiotoxicity caused by DOX is controversial and extensive research has been done on the topic. When congestive heart failure develops
post-DOX use, mortality is approximately 50% in one year. One of the many strategies to reduce or eliminate DOX toxicity involves the encapsulation of DOX within different materials in order to increase the drug specificity (targeting to cancer cells) and thus, reduce its side effects.

### 2.2.3. Encapsulation of anticancer drugs

Encapsulation of anticancer drugs within different macromolecules, nanoparticles, or even stem cells has become a main strategy to reduce side effects and/or increase selectivity. Some of the current materials that are under investigation include liposomes, micelles, dendrimers, nanoparticles, magnetic nanoparticles, carbon nanotubes, graphene, polypolymeric-drug conjugate nanoparticles, lipid-based drug carriers, ceramic-based nanoparticles, and hydrogels among others. Of these, magnetic nanoparticles, such as superparamagnetic iron oxide nanoparticles (SPIONs), have the potential to be (1) used as drug carriers, (2) visualized in vivo by MRI, and (3) manipulated by an external magnetic field. In such magnetic systems, therapeutic compounds, are covalently or non-covalently linked to magnetic nanoparticles and strong magnetic fields are focused on specific target sites in vivo, resulting in enhanced delivery to the site of interest.
Many anticancer drugs have been encapsulated to improve their activity and reduce side effects and one of the drugs that is particularly attractive for this application is DOX. Due to the inherent fluorescence that DOX possesses, it is relatively easy to quantitatively determine the degree of drug-encapsulation in the material of interest. Specifically, many formulations containing DOX and magnetic nanoparticles (for magnetic targeting and MRI tracking) have been reported. Some examples include the co-encapsulation of DOX and iron-cobalt, or Fe$_3$O$_4$ (magnetite) nanoparticles in biodegradable microcarrier capsules with a diameter ranging from ~ 200 nm to more than 50 µm. Others include porous FePt capsules of ~ 340 nm in diameter coated with a lipid membrane and with pores of 20 nm where DOX is encapsulated, Fe$_3$O$_4$ nanoparticles coated with DOX and PEG and functionalized with a porous silica shell, zeolite-Fe$_3$O$_4$ nanocomposites encapsulating DOX, hydrogel nanospheres and nanocomposites containing Fe$_3$O$_4$ nanoparticles.
and DOX, polymeric complexes encapsulating DOX and magnetic nanoparticles, among others.

2.2.4. Heparin – DOX complex

The macromolecular complex of DOX and heparin was first reported in 1978 by M. Minozzi and F. Arcamone. The study reported the ionic interaction between DOX, which has one positive charge (a protonated amine) in aqueous solution, and heparin, which has a highly negative charge (Figure 2.10). Heparin is a naturally-occurring glycosaminoglycan that has been used for decades as an anticoagulant to prevent the formation of blood clots and to treat existing ones. The strong interaction between these two clinical agents became popular in the scientific field and many researchers studied the chemical and physical properties of the complex with the aim of developing an anticancer agent with prolonged action and reduced side effects. A later study evaluated the chemotherapeutic potential of the DOX-heparin complex in vitro, in leukemic cells, and in vivo, in tumor-bearing mice, concluding that the complex decreased the acute and chronic cardiotoxicity of DOX without reducing the antitumor activity in mice.

Figure 2.10 – Chemical structure of heparin.
2.2.5. Ferumoxytol (Feraheme®)

Ferumoxytol is a superparamagnetic iron oxide nanoparticle with a polyglucose sorbitol carboxymethylether coating manufactured by AMAG Pharmaceuticals (Waltham, Massachusetts, USA) under the trade name of Feraheme®. Ferumoxytol was approved by the United States Food and Drug Administration (FDA) on June 30, 2009 for the treatment of iron-deficiency anemia in adult patients, a condition that develops where there is a low number of oxygen-carrying red blood cells because of low iron concentrations. Ferumoxytol is given by i.v. administration, and it is sold in a concentration of 30 mg/mL of elemental iron (510 mg in 17 mL). The molecular weight of ferumoxytol has been reported to be 750 kDa with an osmolality of 291 mOsmol kg⁻¹, a diameter of about 17 to 31 nm, and the following chemical formula: Fe_{5874} O_{8752} C_{11719} H_{18682} O_{9933} Na_{414}. Besides being used to treat anemia, ferumoxytol has been studied and used in clinical settings as an MRI CA.
Chapter 3

Bi@US-tubes as an intracellular contrast agent

3.1. Introduction

Stem cell-based therapies represent a promising future for regenerative medicine, oncology, and other medical fields. Imaging techniques provide a means for non-invasive monitoring and tracking of in vivo transplanted stem cells. X-ray computed tomography (CT) is the most frequently used diagnostic imaging technology in the clinic to detect a wide range of diseases, anatomical abnormalities, and others. Micro-computed tomography (micro-CT) has emerged in recent years as a powerful and non-invasive preclinical imaging

tool used to produce high-resolution images (higher than clinical CTs) with microscopic resolution (voxel size < 100 μm³), and scan times ranging from minutes to tenths of minutes. At present, CT is not considered to be a cellular imaging modality due to the lack of CAs that are either cell-permeable or surface-modified so that they can bind selectively to receptors on the cell exterior.

US-tubes have proven to safely internalized into stem cells while delivering Gd³⁺ ions to render the cells MRI-active. As an analogous material, US-tubes have been used to encapsulate and transport Bi³⁺ ions as a new concept in CT CA design for the internal labeling of stem cells. The encapsulation of bismuth as BiOCl/Bi₂O₃ within US-tubes (Bi@US-tubes) has been previously achieved and the material was characterized by high-resolution transmission electron microscopy (HR-TEM), energy-dispersive X-ray spectroscopy (EDS), thermogravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS), and Raman spectroscopy. Bi@US-tubes were used for intracellular labeling of pig bone marrow-derived mesenchymal stem cells (MSCs), delivering 10⁷ Bi³⁺ ions per cells, allowing cellular imaging with an X-ray-based modality for the first time. The X-ray contrast was achieved with low bismuth loading (2.66% by weight) within the US-tubes.

The Bi@US-tubes material was well-characterized prior to be used as an intracellular CA to label MSCs. The labeled MSCs showed higher X-ray attenuation compared to control cells when imaging the samples using a micro-CT (Figure 3.1). However, the performance of the MSCs after the internalization of the Bi@US-tubes was not studied in depth. This chapter aims to present a detailed study of the MSCs properties
post-labeling to further evaluate whether the Bi@US-tubes affects the normal behavior of the cells, and thus, if it can potentially be used as an intracellular CA for further studies.

Figure 3.1 – (A) HR-TEM image of the solid Bi@US-tube material, yellow arrows indicate the presence of bismuth as confirmed by EDS. (B) Photograph of the unlabeled MSCs and Bi@US-tube-labeled MSCs, (C) CT image of the cell pellets, and (D) CT images using the colored mode. Adapted from Ref. 8.

3.2. Materials & Methods

3.2.1. Synthesis of the Bi@US-tubes

Bi@US-tubes were prepared as described elsewhere. Briefly, US-tubes were prepared by the fluorination method, followed by pyrolysis (more details in Chapter 4, page 40). BiCl₃ (15 mg) was first dissolved in 15 mL of HPLC water in a scintillation vial
to produce an opaque white solution, due to the formation of BiOCl. 70% HCl was then added dropwise to the solution with vigorous stirring until the color changed from white to colorless. 15 mg of US-tubes were added to the solution and then sonicated for one hour. The Bi@US-tubes were collected by filtration, washed with abundant diluted acid (1 M HCl) to remove free bismuth ions, and then with abundant distilled water. The resulting Bi@US-tube sample was then dried at 120 °C overnight.

A stock labeling solution was made by suspending the Bi@US-tubes in 0.17% (w/v) Pluronic® F-108 via probe sonication for 5 min, followed by centrifugation at 3200 rpm for 10 min to remove any unsuspended Bi@US-tubes. Pluronic® F-108 is a non-ionic, non-cytotoxic surfactant with an average molecular weight of 14.6 kDa that is commonly used to suspend CNTs and other nanomaterials for biological applications. The stability of the Bi@US-tube suspension in different biological mediums was examined before, and showed no leaking of the Bi\(^{3+}\) ions in biological-relevant mediums. For the cell labeling studies, the Bi\(^{3+}\)-ion concentration of the stock solution was maintained from 60 to 66 µM, as confirmed by inductively-coupled plasma-optical emission spectrometry (ICP-OES, Optima 4300 from PerkinElmer, Inc.). The stock solution was sterilized under UV light for 3 h prior to its addition to cell cultures.

### 3.2.2. Labeling of MSCs

MSCs were harvested and isolated from the bone marrow of at least three different pigs, and expanded in two successive passages at 500 cells/cm\(^2\). Cells in the second passage (P\(_2\)) were frozen in cryovials and then thawed and expanded (P\(_3\)) prior to labeling. Cell cultures were incubated at 37 °C (95% relative humidity in 5% CO\(_2\)). MSCs were grown
and maintained in alpha-minimum essential medium (αMEM) containing 10% fetal bovine serum (FBS). All labeling studies were performed with P3 MSCs. MSCs were labeled with Bi@US-tubes, to have a final concentration of 24 µM Bi^{3+}. After 24 h, the cells were washed with phosphate buffered saline (PBS) three times and exposed to trypsin-EDTA for ~5 min at 37 °C. The cell suspension of Bi@US-tube-labeled MSCs was then passed through a 70 µm nylon filter and transferred to centrifuge tubes. A density gradient centrifugation technique was performed to isolate Bi@US-tube-labeled MSCs from the excess Bi@US-tubes in solution. Briefly, Histopaque® 1077 (25 °C, Sigma-Aldrich) was slowly added in a 1:2 volume ratio (Histopaque:cells) to the bottom of the tube and centrifuged at 400 × g for 20 min. Cells were collected from the interface of the two liquids, washed with αMEM and centrifuged at 1500 rpm for 10 min. When required, cells were counted using a Beckman Coulter MultiSizer 3.

3.2.3. Viability/cytotoxicity of MSCs

Bi@US-tube-labeled MSCs were prepared as described above. Positive control (unlabeled MSCs) and negative control (dead unlabeled MSCs, treated with 70% methanol for 20 min at 37 °C) were also studied. To determine the viability of the Bi@US-tube-labeled MSCs, a LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen™) was used. The kit consists of two reagents: calcein AM, which fluoresces green when cells are viable, and ethidium homodimer-1 (EthD-1), which fluoresces red when the cellular membrane is compromised. The reagents were added to each sample and cells were incubated in the dark for 20 min at room temperature. Fluorescence-activated cell sorting (FACS, BD Excalibur Flow Cytometer) was used to measure fluorescence intensities.
3.2.4. Population doubling time (PDT) assay

MSCs were cultured with Bi@US-tubes (24 μM Bi³⁺) for 24 h and handled as described above. Bi@US-tube-labeled MSCs, as well as cells treated with 0.17% Pluronic®, and unlabeled cells, were replated on 96-well tissue culture plates at 1×10^3 cells/well. The proliferative activity was measured for up to 144 h using a CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen™), which provides accurate cell numbers based on DNA content. Every 24 h the culture medium was replaced with the CyQUANT reagent, cells were incubated for 1 h at 37 °C, and fluorescence was measured using a microplate reader (TECAN Safire 2™) at 485/528 nm (excitation/emission). In order to calculate the cell number corresponding to the fluorescence intensities, a standard curve was prepared by plotting fluorescence against known numbers of MSCs (unlabeled cells, cells exposed to Pluronic®, and Bi@US-tube-labeled MSCs). PDT values were calculated using the formula:

\[
PDT = T \left( \log \left( \frac{X_E}{X_B} \right) \right) ^{-1}
\]

**Equation 3.1 – Formula for population doubling time (PDT)**

Where \( T \) is the incubation time in hours, \( X_E \) represents the cell number at the end of the incubation time and \( X_B \) corresponds to the cell number at the beginning of the incubation time. PDT values represent an average from a wide range of cell division rates within the culture.
3.2.5. Colony-forming unit fibroblast (CFU-F) assay

The ability of MSCs to self-renew upon Bi@US-tubes exposure was also investigated. Bi@US-tube-labeled MSCs were plated in 75 cm² tissue culture flasks at 1500 cells/flask (20 cell/cm²). The low plating density allows cells to grow as individualized colonies. For the CFU-F enumeration, triplicate flasks were plated and cultured for 14 days with medium replacement every 3 to 4 days. Flasks were then washed with PBS, fixed with 70% methanol, and stained with Geimsa. Unlabeled MSCs and MSCs treated with 0.17% Pluronic® for 24 h were also plated under the same conditions and used as controls. Colony-forming units contain from hundreds to thousands cells which allow them to be seen colonies were counted under a stereomicroscope.

3.2.6. Cell differentiation

The potential of MSCs to differentiate into chondrocytes, osteocytes and adipocytes was evaluated after the cells were labeled with Bi@US-tubes. Labeling was performed by 24 h incubation of cell cultures with Bi@US-tubes (24 μM Bi³⁺). Unlabeled cells were used as controls. Cultures were maintained in differentiation medium for 14 – 21 days.

For adipogenic and osteogenic differentiation, MSCs were initially plated and grown in 6-well tissue culture plates at 20×10³ cells/well in αMEM. After 24 h, the culture medium in the plates prepared for osteogenic differentiation was replaced with the differentiation medium (αMEM supplemented with 10% FBS, 50 μg/mL ascorbate 2 phosphate, 0.1 μM dexamethasone, and 10 mM β-glycerol phosphate), and every 3 to 4 days, fresh differentiation medium was added to cultures. Upon completion, cells were
stained with Alizarin Red S, which showed a bright red color indicating the presence of extracellular calcium accumulation, which is a characteristic of osteocytes activity.

Once the cell confluence reached 50% in plates prepared for adipogenesis induction, adipogenic differentiation medium (αMEM supplemented with 10% FBS, 1% insulin-transferrin-selenium (ITS), 1 μM dexamethasone, 0.5 mM methyl-isobutylxanthine, and 100 μM indomethacine) was added to the cultures. The MSCs were kept in the differentiation medium for three days and subsequently in adipogenic maintenance medium (αMEM supplemented with 10% FBS and 1% ITS) for the following 48 hours. This procedure was repeated twice. The adipogenic cultures were stained with Oil Red O and counterstained with Hematoxylin to stain the lipid vacuoles in red and cell nuclei in blue, respectively.

For chondrogenic differentiation, 200×10³ MSCs were transferred into a 15 mL conical tube and centrifuged at 1200 rpm for 5 min, followed by careful removal of the supernatant. Cell pellets were then incubated in chondrogenic differentiation medium (αMEM supplemented with 1% ITS, 40 μg/mL proline, 100 μg/mL sodium pyruvate, 0.1 μM dexamethasone, 50 μg/mL ascorbate 2 phosphate, 10 ng/mL TGF-β3, and 0.2 μg/mL BMP6) for 21 days. Differentiation medium was replaced every 3 to 4 days. During this time, the MSCs formed a pellet at the bottom of the conical tube in a spherical form that, upon completion of the assay, was fixed with 4% paraformaldehyde, embedded in paraffin, and stained with Alcian Blue to reveal accumulation of glycosaminoglycans, an abundant component of cartilaginous tissue.
3.2.7. Statistical analysis

All experiments were conducted in triplicate and data is reported as mean ± standard deviation. Statistical analyses were performed using GraphPad Prim 7.00. Single-factor analysis of variance (one-way ANOVA) test was used to determine statistical significances and the level of significance, alpha, was defined at 5%.

3.3. Results & Discussion

3.3.1. Cytotoxicity of the Bi@US-tubes

The clinical applications of many CAs are limited by their toxicity. The cell viability of unlabeled MSCs and Bi@US-tube-labeled MSCs was obtained from the fluorescence intensities of calcein AM and EthD-1 using FACS analysis. Unlabeled MSCs (positive control) showed that more than 98% of the viable cells were calcein-positive. MSCs were successfully labeled with Bi@US-tubes (24 μM Bi$^{3+}$), without affecting cell viability for 24 hours (99% life cells based on calcein AM from the LIVE/DEAD assay). Cell viability showed no statistical significant difference (p > 0.05) between the controls and labeled MSCs.

Figure 3.2 shows the gating for the FACS data. Since samples contain just one type of cell (MSC), the gating excludes non-viable cells (debris) or clusters of cells, thus the analyzed population consisted of viable single cells. Four different experimental conditions are shown in Figure 3.2; (1) unlabeled cells with no staining, (2) unlabeled cells stained with calcein and EthD-1 (positive control), (3) dead cells stained with calcein and
EthD-1 (negative control), and (4) Bi@US-tube-labeled MSCs stained with calcein and EthD-1. Positive control cells stained with just calcein and negative control cells stained with just EthD-1 were also analyzed, as well as auto-fluorescence (no staining) of unlabeled and labeled cells (data not shown).

**Figure 3.2** – Data obtained by fluorescence-activated cell sorting (FACS) of unlabeled MSCs with no dyes (unstained cells), positive control cells (unlabeled live MSCs), negative control cells (unlabeled dead MSCs), and Bi@US-tube-labeled MSCs. Representation of the gating for the FACS data; (A) gating of the single cells, (B) gating of the viable cells, and (C) diagram of the side-scattered light (SSC) and Allophycocyanin (ACP-A) of the viable cell population.
3.3.2. Growth kinetics

To evaluate if the Bi@U-tubes affect the growth pattern, proliferation kinetics, and self-renewal properties of the MSCs, PDT and CFU-F assays were performed on unlabeled MSCs, Bi@US-tube-labeled MSCs, and cells treated with 0.17% Pluronic®. The PDT assay provides information about the kinetics growth of the MSCs based on the cell number by detecting the amount of DNA present in the sample (refer to section 3.2.4.). Figure 3.3 shows the proliferation pattern of the three different MSC samples. No significant difference in five (out of six) of the time points was found between the unlabeled MSCs, MSCs treated with Pluronic®, and Bi@US-tubes. However, there is a slight decrease in the number of cells at 48 h for the Bi@US-tube-labeled MSCs and Pluronic®-treated MSCs, which is expected during the lag phase. Furthermore, the growth rate of the Bi@US-tube-labeled MSCs during the exponential phase was equivalent to control cells and reached a significantly higher number of cells by 120 h compared to control cells (p = 0.037), suggesting that the Bi@US-tubes might promote a faster growth. The PDT values obtained from this assay are shown in Table 3.1. PDT values indicate the time (in hours) that takes for a cell population to doubled their number of cells. As shown in Table 3.1, MSCs divided every 19 - 24 h, which has been reported before for porcine MSCs derived from bone marrow.189
Figure 3.3 – Proliferation pattern of control cells, MSCs treated with Pluronic®, and Bi@US-tube-labeled MSCs. # indicates $p < 0.5$ when compared to unlabeled MSCs (control). Results reported in mean ± SD.

Table 3.1 – Population doubling time (PDT) values of unlabeled MSCs (control), and MSCs treated with Pluronic® and Bi@US-tubes. Results reported in mean ± SD, $p > 0.05$.

<table>
<thead>
<tr>
<th>MSCs</th>
<th>hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.3 ± 3.1</td>
</tr>
<tr>
<td>Pluronic®</td>
<td>24.8 ± 8.4</td>
</tr>
<tr>
<td>Bi@US-tubes</td>
<td>19.9 ± 2.6</td>
</tr>
</tbody>
</table>
MSCs clonogenic activity is measured by CFU-F numbers, which provides evidence of the MSCs potency and it can serve as a validation for the PDT assay since it provides information about the MSCs proliferative capability. Figure 3.4 shows the number of colonies for Bi@US-tube-labeled MSCs, Pluronic®-treated MSCs, and unlabeled cells, after being cultured in αMEM for 14 days. The average number of colonies among the three samples did not differ, showing that the self-renewal ability of the MSCs was not affected after the intracellular labeling of the Bi@US-tubes, or the exposure to Pluronic®.

![Bar graph showing number of colonies for control MSCs, Pluronic®-treated MSCs, and Bi@US-tube-labeled MSCs. Results reported in mean ± SD, p > 0.05.]

3.3.3. Cell differentiation

Many of the therapies involving stem cells are based on the unique characteristic of these cells to differentiate into other types of cells, opening a wide range of possibilities
for regenerative medicine and other clinical fields. Therefore, it is crucial to evaluate the
differentiation potential of the MSCs after exposing them to a foreign material, in this case
Bi@US-tubes. MSCs are stromal multipotent adult stem cells that have the ability to
differentiate into progenitor cells of adipose, bone and cartilage tissues. To induce
differentiation, MSCs were incubated with the respective differentiation-inducing media
for prolonged times (> 2 weeks) as described in Section 3.2.6. Figure 3.5 shows the images
for the differentiated MSCs.

As shown in the figure, Bi@US-tube-labeled MSCs are able to differentiate into
the three described lineages as the control cells. MSC cultures were stained to show the
storage of lipids (adipogenic), accumulation of calcium (for osteogenic), and
glycosaminoglycans polysaccharides (for chondrogenic). For the chondrogenic samples
(Figure 3.5C), it is possible to identify the CNT material in the sample, which appear as
black agglomerates at the center of the cell pellet. In addition, the Bi@US-tube material
can be seen in fully differentiated chondrocytes located in the periphery of the cell pellet.
These findings confirm that the direct exposure to CNTs for ~ 21 days did not affect the
MSCs ability to differentiate into chondrocytes.
Figure 3.5 – Histochemical stains of unlabeled MSCs and Bi@US-tube-labeled MSCs. (A) Adipogenic differentiation; the presence of lipid vacuoles appear in red, stained with Oil Red O. (B) Osteogenic differentiation; the presence of calcium accumulations was stained with Alizarin red. (C) Chondrogenic differentiation; Alcian blue stains the presence of glycosaminoglycans. Scare bar = 50 μm.
3.4. Conclusion & Future Directions

In summary, this chapter presents the evaluation of MSCs after the intracellular labeling of the cells with Bi@US-tubes, an X-ray CA. The Bi@US-tube material consists of US-tubes loaded with Bi$^{3+}$ ions (∼ 2.6 % by weight), and its synthesis, characterization, and performance as a radiocontrast agent has been studied and reported previously. Here, the viability, proliferation and differentiation properties of Bi@US-tube-labeled MSCs were studied for the first time and compared to control unlabeled MSCs with the aim to validate the use of Bi@US-tubes as an intracellular CA for stem cell imaging. The results showed no major significant differences between Bi@US-tube-labeled MSCs and control cells. However, a more accelerated growth in cultures of Bi@US-tube-labeled MSCs compared to control cells was observed. This finding suggests that Bi@US-tubes may affect cell proliferation.

One of the limitations of using radiocontrast agents to track stem cells in vivo is the high concentrations of CA needed to obtain a significant X-ray contrast. Therefore, a CA containing higher concentrations of bismuth will be needed and a similar evaluation of its effects in vitro must be conducted before moving to pre-clinical animal studies. One such agent, called Bi$_4$C@US-tubes, which is a second generation material of the Bi@US-tube concept, is described in Chapters 4 and 5 of this thesis.
4.1. **Introduction**

CNTs have been used for a plethora of biomedical applications including their use as delivery vehicles for drugs, imaging agents, proteins, DNA, and other materials. Here we describe the synthesis and characterization of a new CNT-based CA for CT imaging. The CA is a hybrid material derived from US-tubes and Bi(III) oxo-salicylate clusters with four Bi(III) ion per cluster (Bi$_4$C). The element bismuth was chosen over iodine, which is the conventional element used for radiocontrast agents in the clinic today, due to its high X-ray attenuation capability and its low toxicity (refer to Section 2.1.7, page 14 for more details). Bismuth has been used for a number of drug and cosmetic formulation,\textsuperscript{190–192} and its safety has been extensively evaluated,\textsuperscript{99,100} which make bismuth a promising element for new CT CA design. Furthermore, CNT-based CAs containing bismuth have showed to
be a promising intracellular agent for the labeling and imaging of stem cells, as reported
on Chapter 3. This new CA termed Bi$_4$C@US-tubes, was produced with the aim of
increasing the bismuth content in US-tubes (higher than the 2.66% obtained with the
Bi@US-tubes) in order to deliver a greater concentration of this heavy metal
intracellularly, and therefore obtain better X-ray contrast.

The Bi$_4$C@US-tube nanomaterial contains 10× more bismuth than the first
generation material Bi@US-tubes. This chapter presents the preparation and
characterization of Bi$_4$C@US-tubes and its ability to attenuate X-rays compared to empty
US-tubes, and its pioneer material Bi@US-tubes.

4.2. Materials & Methods

4.2.1. Preparation of Bi$_4$C@US-tubes

US-tubes were produced by first cutting SWCNTs (> 1 μm, Carbon Solution, Inc.)
via a fluorination/pyrolysis method described elsewhere. Briefly, 600 mg of SWCNTs
were exposed to 2% F$_2$ in a He gas mixture simultaneously with a flow of H$_2$ gas, both at
a flow rate of 15 cm$^3$/min at 125 °C for ~10 h. A 30 to 40% weight increase was obtained
after the fluorination process. The fluorinated SWCNTs were then pyrolyzed at 1000 °C
for 2 h under constant Ar flow. The produced US-tubes were then purified by sonication
in concentrated HCl$_{(aq)}$ for 1 h (1:1 ratio of US-tubes (mg) to HCl$_{(aq)}$ (mL)), to remove
metal impurities. The purified US-tubes were then individualized by Na$^0$ reduction in dried
THF for 1 h with bath sonication. Finally, the individualized US-tubes were refluxed in 6
M HNO$_3(aq)$ for 10 min to functionalize the ends and defect sites with carboxylate groups.
Finally, the US-tubes were extensively washed with distilled water and dried at 120 °C overnight.

Synthesis of the [Bi₄(μ₃-O)₂(HO-2-C₆H₄CO₂)₈] · 2MeCN cluster (Bi₄C, Figure 4.1), a tetranuclear oxo cluster with a planar Bi₄O₂ core, was performed by mixing BiPh₃ with salicylic acid in wet acetonitrile and left under vacuum for 48 h for solvent removal as described elsewhere.²⁹⁴ 20 mg of the Bi₄C cluster were dissolved in 20 mL of either dried tetrahydrofuran (THF), ethanol (EtOH) or dimethyl sulfoxide (DMSO), and 20 mg of US-tubes were added to the mixture followed by bath sonication for 1 h. The resulting material was washed with 400 mL of the respective solvent used (THF, EtOH, or DMSO) to remove any free Bi₄C, followed by 1.6 – 2.0 L of distilled water to remove the organic solvent from the material. Lastly, the produced Bi(III) oxo-salicylate cluster@US-tube (Bi₄C@US-tube) material was dried overnight at 120 °C for characterization.

Figure 4.1 – (A) Crystal structure of [Bi₄(μ₃-O)₂(HO-2-C₆H₄CO₂)₈] · 2MeCN (Bi₄C, MW: 2046.91) and (B) the structure of the Bi₄ core. Reprinted with permission from Ref. 193.
4.2.2. Characterization of Bi$_4$C@US-tubes

Nuclear magnetic resonance spectra (NMR, Bruker 400 MHz NMR spectrometer) of the Bi$_4$C cluster was taken before and after sonication in THF for 1 h to determine if sonication modified the structure of the cluster. Samples were sonicated in THF for 1 h, followed by removal of the solvent by rotovaporation. NMR spectra were obtained using d6-DMSO as the solvent.
Atomic force microscopy (AFM, Digital Instruments MultiMode AFM-2) was used to evaluate the length of the SWCNTs after the cutting process. Raman spectroscopy was performed using a Renishaw® inVia Raman Microscope equipped with a 633 nm laser.

The presence of Bi$^{3+}$ ions in the Bi$_4$C@US-tube samples was confirmed by X-ray photoelectron spectroscopy (XPS, PHI Quantera SXMTM) and by ICP-OES. Samples for XPS were prepared by pressing a small amount of the solid material into indium foil. The binding energies acquired by XPS were determined by referencing C 1s to 284.50 eV. To analyze the material using ICP-OES, solid samples were digested and dissolved in aqueous solutions. Briefly, the material was weighted using an analytical microbalance, transferred to scintillation vials, digested with 70% HNO$_3$(aq) trace-metal grade and 26% HClO$_3$(aq), and diluted with 2% HNO$_3$(aq) trace-metal grade. A solution of 5 ppm Y$^{3+}$ was used as the internal standard during the analysis. Prior to this, different acids were used for the digestion of a Bi$^{3+}$ standard to determine which one provides the most complete digestion of the organic matter in the sample without affecting the Bi$^{3+}$ concentration. As a validation step, the recovery of Bi$^{3+}$ was obtained by placing 100 µL of a 1000 ppm Bi$^{3+}$ plasma standard solution (Specpure®) in cantillations vials, followed by the addition of 1mL of either 70% HNO$_3$, 26% HClO$_3$, or a combination of both acids. Final Bi$^{3+}$ concentrations were obtained by ICP-OES and the percent recovery was calculated.

Thermogravimetric analysis (TGA) was performed using a SDT Q600 instrument. TGA experiments were conducted under a flow of air at 100 mL min$^{-1}$ with a heating rate of 10 °C min$^{-1}$ from 50 to 800 °C. Electron microscopy techniques were utilized to examine the structure of Bi$_4$C@US-tubes. High-resolution transmission electron microscopy (HR-
TEM) experiments were performed using a JOEL 2100 field emission gun TEM equipped with energy-dispersive spectroscopy (EDS) operated at 200 kV. Samples were prepared by suspending the material in water, followed by placing a drop of the dispersed material onto a Lacey carbon copper grid. Scanning transmission electron microscope (STEM) images were obtained using a Hitachi SU8230 scanning electron microscope (SEM) instrument equipped with a Bruker Flatquad EDS. STEM experiments were performed by depositing a drop of a suspension of the Bi₄C@US-tube material suspended in water onto a TEM Lacey carbon copper grid operated at 30 kV.

**4.2.3. Biological stability studies**

The biological stability of the Bi₄C@US-tube material was explored by challenging the material with biological-relevant media. Solid samples of Bi₄C@US-tubes were placed in centrifuge filter tubes containing either phosphate-buffered saline (PBS) or α-minimum essential medium (αMEM) with 10% fetal bovine serum (FBS). The samples were kept at 37-40 °C for 24 and 48 h, followed by centrifugation at 1200 rpm for 10 min. The supernatant was analyzed for Bi³⁺ by ICP-OES.

**4.2.4. X-ray CT**

The ability of Bi₄C@US-tubes to attenuate X-rays was examined using a clinical CT scan (iCT 256 Phillips) at St. Luke’s Baylor Hospital, Houston, TX. Solid samples of: (1) empty US-tubes, (2) Bi@US-tubes (the first generation CNT-material containing 2-3% bismuth by weight), and (3) Bi₄C@US-tubes were each placed in separate 1.5 mL Eppendorf tubes. 500 μL of 5% agar were added on top of the solid samples to kept the
samples at the bottom of the centrifuge tubes and avoid static of the solid material with the plastic. Data was acquired using the following scanner parameters: tube voltage = 120 kV, pitch = 0.664, gantry rotation time = 0.33 s, mAs/mA = 150/302, reconstruction slice thickness of 0.625/0.312 cm. For quantitative analysis, three areas per sample were selected as regions of interest (ROI) and measured in Hounsfield units (HU). The reported values are the average measured for axial and coronal views. Analysis was performed using Osirix v. 4.1.2. 32 bit, an open source software.

4.3. Results & Discussion

4.3.1. Characterization of US-tubes

Bi₄C@US-tubes is a new X-ray CA material which consists of a hybrid material containing US-tubes and Bi(III) oxo-salicylate clusters (Bi₄C) which are likely located on at the defect sites of the US-tubes, which are produced by a chemical cutting process as described in Section 4.2.1., page 40. The resulting oxidized US-tubes have a length of 20 – 80 nm as confirmed by AFM (Figure 4.3), and possess defect sites that the pristine full-length SWCNTs lack. The presence of defect sites in the US-tube sidewalls is confirmed by Raman spectroscopy (Figure 4.4), where the D band at ~1300 cm⁻¹ is significantly larger than that of the full-length SWCNTs. The D band is often referred to as the disorder band or defect band, and it indicates disorder or rupture of the sp² hybridization of the full-length SWCNT structure. A small D band is also visible in the Raman spectrum of the full-length SWCNTs which is due to the hybridized vibrational mode associate with the SWCNT edges. For the US-tubes, the D band also exhibits a shoulder around 1200 cm⁻¹ which is
referred to as the D4 band. This band has been previously reported and attributed to sp²-sp³ bonds. In Figure 4.4, the small band mark with an asterisk symbol (*) is attributed to the vibrational modes associate to the υ(-C-H) functional groups, which are present in the US-tubes but not in the raw SWCNTs.

Figure 4.3 – AFM images of (A) a bundled of pristine SWCNTs (> 1 μm) and (B) bundles of US-tubes.
Figure 4.4 – Raman spectra of the pristine SWCNTs and US-tubes, (*) is attributed to the vibrational modes associate to the $\nu$(-C-H) functional groups.

4.3.2. Characterization of Bi$_4$C@US-tubes

To synthesize the Bi$_4$C@US-tubes, three different organic solvents were tested: THF, EtOH and DMSO. The presence of bismuth and its oxidation state (+3) was also established by ICP-OES and XPS, respectively. For ICP-OES analysis, the material has to be first digested with strong acids, and an aqueous solution prepared. As a validation step, the recovery of Bi$^{3+}$ was first obtained. As shown in Figure 4.5, the best recovery % was obtained when using HClO$_3$ alone or when HNO$_3$ and HClO$_3$ were used in combination. Therefore, the combination of the two acids was used for all experiments described here.
The highest bismuth concentration was obtained when using THF as the solvent during the sonication step, with the resulting material containing ~20% bismuth by weight and ~3.5% atomic bismuth relative to carbon (Figure 4.6). Therefore, for further experiments, THF was used as the solvent of choice for the preparation of Bi$_4$C@US-tubes. Figure 4.7 shows the high-resolution XPS spectrum for Bi$_4$C@US-tubes, where the two intense bands in the bismuth region are assigned to Bi 4f 7/2 and Bi 4f 5/2, which are the distinctive bands for Bi$^{3+}$.\textsuperscript{197,198}
Figure 4.6 – The concentration of $\text{Bi}^{3+}$ by (A) ICP-OES, and (B) XPS of $\text{Bi}_4\text{C}@@\text{US}$-tubes when prepared in three different solvents.

Figure 4.7 – The characteristic XPS bands for $\text{Bi}^{3+}$ when using THF as the solvent for the preparation of $\text{Bi}_4\text{C}@@\text{US}$-tubes.
$^1$H NMR was used to investigate whether the rigorous sonication process used to prepare the Bi$_4$C@US-tube sample affects the structure of the Bi$_4$C cluster. For this, the $^1$H NMR spectrum of the Bi$_4$C cluster before and after its sonication in THF was attained. The two spectra showed no major differences, however, after sonication, the methyl signal for acetonitrile was not observed (Table S1), suggesting that acetonitrile, which is a weak ligand in the parent cluster, is displaced by another ligand, probably THF or water, after sonication of THF (Table 4.1).

Table 4.1 – $^1$H NMR spectra for the Bi(III) oxo-salicylate cluster (Bi$_4$C) before and after sonication in THF.

<table>
<thead>
<tr>
<th>Bi$_4$C dissolve in THF $\delta$ (ppm)</th>
<th>Bi$_4$C sonicated in THF $\delta$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.9 (s, 1H, PhOH),</td>
<td>11.9 (s, 1H, PhOH),</td>
</tr>
<tr>
<td>7.67 (s, 1H, o-H),</td>
<td>7.67 (s, 1H, o-H),</td>
</tr>
<tr>
<td>7.31 (s, 1H, p-H),</td>
<td>7.31 (s, 1H, p-H),</td>
</tr>
<tr>
<td>6.80 (s, 2H, m-H),</td>
<td>6.80 (s, 2H, m-H),</td>
</tr>
<tr>
<td>2.07 (s, 1H, CH$_3$)</td>
<td>2.45 (s, 1H, DMSO)</td>
</tr>
<tr>
<td>2.45 (s, 1H, DMSO)</td>
<td>1.74 (m, 2H, THF)</td>
</tr>
<tr>
<td>1.74 (m, 2H, THF)</td>
<td></td>
</tr>
</tbody>
</table>

The Raman spectrum of Bi$_4$C@US-tubes was also obtained and compared with the spectrum of the US-tubes (Figure 4.8). The D band is at slightly lower intensity for Bi$_4$C@US-tubes, which is due probably to the association of the Bi$_4$C cluster with the US-tubes sidewall defects, which perturbs the natural vibrational modes associated with the edges and the defect sites of the US-tubes. The G’ band also differs between the two materials. This is due because the G’ band is the second overtone of the D band, and
therefore a change in the G’ band is expected when the D band is altered, although its intensity is often greater than that of the D band. The Raman spectrum of the Bi$_4$C@US-tube sample also showed the small shoulder denominated as the D4 band, as well as another shoulder around 900 cm$^{-1}$ that the US-tubes do not show. This band of low intensity has been associated with C-O-C vibrational modes, which are present for the Bi$_4$C cluster.

The radial breathing mode (RBM) band, which appears around 200 cm$^{-1}$ and can be used to determine the diameter of the CNTs, rise from the natural vibration of the CNT diameter contracting and expanding. As seen in the figure, the RBM band does not differ between the two spectra, which is expected since the nature of the US-tube was not modify by adding the Bi(III) oxo-salicylate cluster during the synthesis process of Bi$_4$C@US-tubes.

![Raman spectra](image)

**Figure 4.8** – Raman spectra of US-tubes and Bi$_4$C@US tubes. * Indicates the C-O-C vibrational modes which are present in the Bi(III) oxo-salicylate cluster.

TGA was used to further analyse the thermochemical and physical properties of Bi$_4$C@US-tubes. Figure 4.9 displays the characteristic TGA curve in air of (1) US-tubes,
as well as the TGA curves of (2) the Bi₄C alone, (3) Bi₄C@US-tubes and (4) a mixture of equivalent amounts of Bi₄C and US-tubes (Bi₄C/US-tubes). The TGA data shows that US-tubes are thermally stable up to ~500 °C, where the sample starts to show a significant mass loss. The Bi₄C cluster is stable up to ~160 °C, after which the first loss of mass takes place probably due to the elimination of one of the ligands, whereas the remaining ligands are removed at 250 – 350 °C with complete decomposition occurring at ~350 °C. This TGA pattern has been reported before for similar Bi³⁺ compounds synthetized when BiPh₃ is mixed with 3-hydroxy picolinic acid, a derivative of salicylic acid, under different conditions.²⁰⁰ In contrast, the Bi₄C@US-tubes sample is thermally stable up to ~340 °C, with just one mass loss, indicating that the US-tubes and Bi₄C discompose simultaneously as one single material.

For the case of having the Bi₄C cluster and the US-tubes being present as a physical mixture, several mass losses were obtained, as showed in Figure 4.9. Sudden mass losses corresponding to the Bi₄C cluster components were observed around 160, 260 and 350 °C, with final decomposition occurring around 450-500 °C due to the degradation of the US-tubes.
Figure 4.9 – TGA curves of empty US-tubes, Bi(III) oxo-salicylate cluster (Bi$_4$C), and Bi$_4$C@US-tubes in air. At the top right is the TGA decomposition pattern for a 1:1 physical mixture of the Bi(III) oxo-salicylate cluster and the US-tubes (Bi$_4$C / US-tubes*). The first derivative is presented (in blue) to better show where the mass losses occur.

TEM images of Bi$_4$C@US-tubes showed the characteristic agglomeration of a carbon nanotubes material in water which form a fiber-like network. High density spots along the US-tubes are remarkably uniform in length (~3-5 nm). The presence of Bi$^{3+}$ ions at these high density spots was confirmed by EDS, as shown in Figure 4.10. None of the high dense spots were found outside of the US-tubes network, demonstrating that Bi$_4$Cs are entirely associated with the US-tubes, which is consistent with the above TGA data.
However, none the TGA or the TEM data revealed definitive evidence as to whether the Bi$_4$C clusters are all along the outer surface of the US-tubes or only at the defect sites.

![TEM images of the Bi$_4$C@US-tubes.](image)

**Figure 4.10** – TEM images of the Bi$_4$C@US-tubes. White arrows indicate the Bi(III) oxo-salicylate cluster, as confirmed by EDS. EDS spectrum is at the bottom.

Furthermore, STEM images showed “bumps” on the surface of the US-tubes, suggesting that the agglomerates of Bi$_4$C are on the outer surface of the US-tubes (Figure 4.11). EDS mapping confirmed that the observed “bumps” contain a high concentration of bismuth, hence, showing that the Bi$_4$C clusters localize on the outer surface of the US-
tubes (Figure 4.12). Both TEM and STEM techniques confirmed the presence of relatively large agglomerates of bismuth, suggesting that the intact Bi₄C cluster, which is 1.7 nm in length and 0.7 nm in diameter forms larger agglomerates on the outer surface of the US-tubes.

Figure 4.11 – STEM images of the Bi₄C@US-tubes tangled in the amorphous carbon film of the sample grid. White arrows point to the Bi(III) – oxo-salicylate clusters. Scale bar = 100 nm.
4.3.3. Biological stability studies

When using Bi₄C@US-tubes as an intracellular CA label, the material is exposed to different microenvironments within or outside of cells. For such applications it is essential that the Bi₄C and the US-tubes remain associate with one another in order to study the cellular localization, behavior and elimination of the Bi₄C@US-tube material. To help determine if the Bi₄C clusters remain associated with the US-tubes in biological environments, the material was exposed to two different biological-relevant media (PBS, and αMEM with 10% FBS), as described in Section 4.2.3, page 44. Bi³⁺ ion was not
detectable in the supernatant by ICP-OES, indicating that the hybrid material is stable in such media for up to 48 h at 37 ºC.

4.3.4. X-ray CT

CT images were obtained using a clinical scanner to evaluate the ability of the Bi₄C@US-tubes to serve as a CT CA. As a control, the X-ray attenuation ability of US-tubes was also determined, as well as the attenuation ability of the previously reported first generation material Bi@US-tubes CA material.⁸ CT images of the solid samples of the three materials are presented in Figure 4.13, in the conventional black-and-white format, as well as in the colored image mode. As reported in Table 4.2 Error! Reference source not found., the new Bi₄C@US-tubes material has an X-ray attenuation performance (HU value) of about 8.3 times higher than that of the first generation Bi@US-tubes CA material, which is expected due to the much higher bismuth concentration in the new CA. The US-tube alone have an HU value of 212 ± 10 which is probably due to the presence of catalytic metal impurities from the synthesis process, which in this case are nickel and yttrium.
Figure 4.13 – CT images (coronal and axial views) of solid samples of (A) US-tubes, (B) Bi@US-tubes, and (C) Bi₄C@US-tubes. Both the black and white images (top), and the colored images (bottom) are presented to better demonstrate performance.

Table 4.2 – Comparative HU values for solid samples of US-tubes, Bi@US-tubes, and Bi₄C@US-tubes.

<table>
<thead>
<tr>
<th>Material</th>
<th>US-tubes</th>
<th>Bi@US-tubes</th>
<th>Bi₄C@US-tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU value</td>
<td>188 ± 12</td>
<td>227 ± 24</td>
<td>2178 ± 309</td>
</tr>
</tbody>
</table>

4.4. Conclusion & Future Directions

This work reports the successful synthesis and full characterization of a new hybrid material (Bi₄C@US-tubes) containing US-tubes and Bi(III) oxo-salicylate clusters, abbreviated as Bi₄C. Bi₄C@US-tubes was characterized by AFM, Raman, ICP-OES, XPS, TGA, TEM and STEM. The hybrid material contains 20% bismuth by weight with the potential to perform as a CA for X-ray-based imaging techniques such as radiography, CT, and fluoroscopy. Its ability to attenuate X-rays has been evaluated using a clinical CT
scanner and compared to empty US-tubes and Bi@US-tubes (reported in Chapter 3), which contained only \( \sim 2.6 \) wt.% of \( \text{Bi}^{3+} \). The X-ray attenuation ability of the new Bi@US-tubes is nearly 8 times greater than that of the first generation material. The stability of the Bi@US-tubes material has been firmly established using biological relevant media challenges (PBS and αMEM containing 10% FBS) which revealed no leaking of \( \text{Bi}^{3+} \) from the hybrid material for up to 48 h at 37 \( ^\circ \)C. However, additional experiments involving the use of different biological-relevant media (varying pH and chemical properties) and longer exposure times (> 48 h) must be performed in order to fully challenge the interaction between the US-tubes and the Bi(III) oxo-salicylate cluster.

The present work continues to demonstrate that the pairing of \( \text{Bi}^{3+} \) and CNT materials can produce new hybrid materials with extremely high X-ray attenuation capabilities. These materials offer the opportunity to develop a new technology to internally label cells with a high concentration of \( \text{Bi}^{3+} \) for the purpose of cellular imaging and potentially \textit{in vivo} cellular tracking by X-ray CT. The labeling and tracking of stem cells by CT would seem to be a most desirable application of this new technology.
Chapter 5

Labeling mesenchymal stem cells with Bi$_4$C@US-tubes for X-ray CT imaging

5.1. Introduction

In stem cell-based therapies, the poor engraftment and survival of the cells represent two major obstacles for the effectiveness of these therapies. Therefore, it is essential to be able to track stem cells \textit{in vivo} to better understand the outcome of stem cell therapies. Numerous researchers are currently working with adult stem cells to treat disease by replacing damaged tissue (in-lineage cell replacement), as well as by providing paracrine survival and growth factors.\textsuperscript{201} Clinical trials involving autologous therapies (where patients receive their own cells) and some allogenic therapies (recipients or hosts receive cells from a donor) have been conducted for diabetes, paraplegia, multiple sclerosis, critical limb ischemia, cerebrovascular disease, blood-related cancers, as well as some solid tumor cancers.\textsuperscript{202} Among the adult stem cells found in the human body, mesenchymal stem cells
(MSCs) have the advantage of being less immunogenic than others, allowing the use of MSCs without concerns about immunological rejection or the need for immunosuppressant drugs. With the rapid increase of reported cases of MSCs-based therapies, there is an urgent need to track the cells in vivo during preclinical and clinical trials to further understand and evaluate their biodistribution, accumulation, and tissue retention.

This chapter presents the in vitro study of Bi$_4$C@US-tubes as an intracellular radiocontrast agent for porcine MSCs. The Bi$_4$C@US-tubes contains 20% by weight of bismuth with no detectable release of bismuth after 48 h exposure to various biological challenges, suggesting the presence of a strong interaction between the US-tube platform and the bismuth clusters.

### 5.2. Materials & Methods

#### 5.2.1. Preparation of the labeling suspension

For in vitro studies, a labeling solution was prepared by suspending the Bi$_4$C@US-tubes in a 0.17% solution of Pluronic®-F108, a non-ionic surfactant, via probe sonication for 5 min. The samples were centrifuged at 3200 rpm for 10 min, and the supernatant was used for the in vitro experiments. The bismuth concentration was determined using ICP-OES and adjusted to 1.0 µM Bi$^{3+}$.

#### 5.2.2. Stability studies

The stability of the Bi$_4$C@US-tubes suspended in 0.17% Pluronic® was assessed to determine if the rigorous sonication process affects the interaction between the Bi$_4$C cluster
and the US-tubes. Aliquots of the labeling solution were placed in centrifuge filter tubes (15 mL volume unit, 30 kDa, Millipore Corp.), kept at 37-40 °C for 24 h and finally centrifuged. The supernatant was treated with aliquots of 70% HNO$_3$(aq) trace-metal grade and 26% HClO$_3$(aq) with heat to digest the organic material (Pluronic®, molecular weight ~14.6 kDa). Samples were analyzed for bismuth by inductively-coupled plasma mass spectrometry (ICP-MS, Elan 9000 from PerkinElmer Inc.).

5.2.3. Labeling MSCs with Bi$_4$C@US-tubes

Porcine MSCs isolated from the bone marrow of adult male pigs (three different animals) were intracellularly labeled with Bi$_4$C@US-tubes. Prior to labeling, the concentration of the labeling solution was determined by ICP-OES and adjusted to 1.0 mM Bi$^{3+}$. To sterilize the labeling solution, the sample was exposed to UV-light for three hours while rocking. MSCs were grown in T-175 flasks with α-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere containing 5% CO$_2$/95% air. Cells were expanded until the third passage and then left to grow until 70-80 % of confluence prior to labeling. The labeling solution was directly added to the culture medium to obtain the desire final concentration. During this process, FBS-free αMEM was used, and FBS was added to the culture flasks four hours post-labeling to obtain a 10% FBS final solution. Subsequently, cells were incubated and left undisturbed for 20 h. Fourteen labeling concentrations, ranging from 20 μM to 300 μM of Bi$^{3+}$ were tested.

For cell collection, MSCs were trypsinized (0.05% trypsin with 0.1% EDTA for ~5 min at 37 °C), and passed through a 70 μm filter to avoid cell aggregates. A density gradient
separation was performed as described elsewhere,\textsuperscript{10,203} to remove free Bi\textsubscript{4}C@US-tubes from the cell suspension. Briefly, cells were re-suspended in 20 mL of αMEM in a 50 mL conical tube and 10 mL of Histopaque\textsuperscript{®} 1077 (25 °C, Sigma-Aldrich) were added slowly to the bottom of the conical tube. Finally, the sample was centrifuged at 400 × g for 20 min. Labeled MSCs were then isolated from the interface of the αMEM and Histopaque\textsuperscript{®} layers using a plastic transfer pipette. Finally, cells were counted using a particle counter (Beckman Counter MultiSizer 3). Unlabeled MSCs were used as control cells and the gradient separation step was not necessary in this case. For all experiments, cells from three different animals were used, and therefore, everything is reported in triplicate.

5.2.4. Elemental analysis of Bi\textsubscript{4}C@US-tubes-labeled MSCs

Aliquots of cell suspensions were collected in glass scintillation vials to determine the bismuth concentration within the cells by ICP-MS analysis. To prepare the samples, cells were heated and treated with two alternating additions of 500 μL 70% HNO\textsubscript{3(aq)} trace metal-grade and 26% HClO\textsubscript{3(aq)}, allowing the samples to dry between additions. Finally, the samples were diluted to 5 mL with 2% HNO\textsubscript{3(aq)} trace metal-grade and filtered through a 0.22 μm pore size syringe filter.

5.2.5. Viability/cytotoxicity of MSCs

Bi\textsubscript{4}C@US-tube-labeled MSCs were prepared as described above. Positive control (unlabeled MSCs) and negative control (dead unlabeled MSCs, treated with 70% methanol for 20 min) were also studied. To determine the viability of the Bi\textsubscript{4}C@US-tube-labeled MSCs, a LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen\textsuperscript{TM}) was used as described
in Section 3.2.3, page 27. Fluorescence-activated cell sorting (FACS, BD Excalibur Flow Cytometer) was used to measure fluorescence intensities.

5.2.6. Electron microscopy

TEM analysis was performed to determine the subcellular localization of the Bi_4C@US-tubes. Labeled MSCs (300 µM Bi^{3+}, for 24 h) and unlabeled MSCs were centrifuged separately at 1500 rpm for 10 min to form a cell pellet (1×10^6 cells/pellet). The supernatant was removed without disturbing the cell pellet and 4% glutaraldehyde was added, after which the samples were left undisturbed for two days at 4 °C. Subsequently, samples were washed with PBS and post-fixed with 1% OsO_4 for 1 h, and then washed and dehydrated with increasing concentrations of ethanol, and then infiltrated with acetone and Epon 812 resin. Finally, samples were embedded in a mold with 100% Epon 812, cut into 1 mm sections, and then stained with 1% methylene blue and 1% basic fuchsin. Ultra-thin sections of 80 nm were cut from the sample block using a Leica EM UC7 ultra microtome and framed on 100-mesh copper grids. Grids were stained with 2% alcoholic uranyl acetate and Reynold’s lead citrate. The grids were examined using a JEOL 1230 TEM instrument equipped with an AMTV 600 digital imaging system at the Texas Heart Institute (Houston, TX).

5.2.7. Label retention

To verify the clearance of the Bi_4C@US-tube material from the MSC cultures, the bismuth content present within cells and in the culture medium were quantified. MSCs isolated from three animals were plated in 25 cm^2 flasks at 2×10^3 cell/cm^2 and allowed to
Flasks were then treated with Bi\textsubscript{4}C@US-tube (300 μM Bi\textsuperscript{3+}) for 24 hours, and the medium was then removed and replaced with fresh αMEM (3 mL). For analysis of bismuth content in the culture medium, two experimental groups were studied: for Group A, fresh media (αMEM) was replaced every subsequent 24 h, while for Group B, the culture media remained unchanged for the remainder of the experiment. To determine intracellular bismuth content, cells from both experimental conditions, Group A and Group B, were collected for the 0 and 72 h time points. Bismuth analysis was performed by ICP-OES.

5.2.8. Population doubling-time (PDT) assay

Bi\textsubscript{4}C@US-tube-labeled MSCs (300 μM Bi\textsuperscript{3+}, 24 h), as well as MSCs treated with 0.17% Pluronic® (24 h), and unlabeled cells, were replated on 96-well tissue culture plates at a density of 1\times10\textsuperscript{3} cells/well. Proliferation of the cells was measured for up to 144 h using a CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen), as described in Section 3.2.4, page 28.

5.2.9. Colony-forming unit fibroblast (CFU-F) assay

Bi\textsubscript{4}C@US-tube-labeled MSCs, Pluronic®-treated MSCs, and unlabeled MSCs were separately plated in 75 cm\textsuperscript{2} tissue culture flasks at 1500 cells/flask. The cells were cultured for 14 days with medium replacement every 3 to 4 days. Flasks were washed with PBS, fixed with 70% methanol, dried, and stained with Geimsa stain. Colonies were counted under a stereomicroscope.
5.2.10. Cell differentiation

To determine if the Bi$_4$C@US-tube material affects the ability of the cells to differentiate into different progenitor cells, unlabeled MSCs and Bi$_4$C@US-tube-labeled MSCs were exposed to differentiation-inducing media to promote adipogenic, osteogenic and chondrogenic differentiation. The differentiation conditions were kept for 14 – 21 days. The assay was performed as described in Section 3.2.6, page 29.

5.2.11. X-ray CT experiments of Bi$_4$C@US-tube-labeled MCSs

The ability of Bi$_4$C@US-tubes to attenuate X-rays once internalized in MSCs was evaluated using a clinical CT scan (iCT 256 Phillips) at St. Luke’s Baylor Hospital, Houston, TX. Bi$_4$C@US-tube-labeled MSCs were prepared as described above, followed by centrifugation for 10 min at 1200 rpm to form a cell pellet (200×10$^6$ cells/pellet). Separately, a cell pellet of control unlabeled MSCs was also prepared in a 1.5 mL Eppendorf tube. 500 μL of 5% agar was added carefully on top of the cell pellets and samples were kept refrigerated at 4 °C. Data was acquired using the scanner parameters described on Section 4.2.4, page 44. For quantitative analysis, three areas per samples were selected as regions of interest (ROI) expressed and measured in Housfield units (HU). The reported values are the average measured for axial, sagittal, and coronal views. Analysis was performed using Osirix v. 4.1.2. 32 bit, an open source software.

5.2.12. Statistical analysis

All experiments were conducted in triplicate and values are reported as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 7.00 and
the single-factor analysis of variance (one-way ANOVA) test was used to determine statistical significances, with the level of significance, alpha, being defined at 5%.

5.3. Results & Discussion

5.3.1. Stability studies

The labeling solution (1 mM Bi$^{3+}$) was prepared as described above. To suspend the Bi$_4$C@US-tubes in a 0.17% Pluronic$^\circledR$ solution, the sample was probe sonicated, a process which might disrupt the interaction between the Bi$_4$C cluster and the US-tubes. To determine if there was leakage of bismuth after sonication, a filtration challenge was performed as described in Section 4.2.2. There was no-detectable bismuth found in the filtered solution, as confirmed by ICP-MS, which demonstrated that the hybrid material remained intact after the rigorous sonication process, and the Bi$_4$C cluster remained bound to the US-tubes.

5.3.2. Labeling of MSCs with Bi@US-tubes

MSCs harvested from the bone marrow of adult male pigs (N=3) were used for the in vitro studies. MSC was chosen as the cell type because they are easily harvested, isolated, expanded in culture, and differentiate in vitro. The stock solution (1 mM Bi$^{3+}$) of the Bi$_4$C@US-tubes suspended in 0.17% Pluronic$^\circledR$ was directly added to the culture flasks. Fourteen different labeling concentrations (10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 140, 160, 180, 200, and 300 μM Bi$^{3+}$) were evaluated in terms of their effect on cell viability and cell uptake of the material. The viability of the MSCs was evaluated by FACS
immediately after cell collection using two dyes: calcein AM for live cells and EthD-1 for dead cells. As seen in Figure 5.1, for the range of concentrations studied, the viability of the MSCs remained greater than 96%, even for the highest concentration (300 µM Bi$^{3+}$), which means that MSCs have a high tolerance toward Bi$_4$C@US-tubes. The cell viability for unlabeled MSCs was 97 ± 3 (positively stained for calcein AM), and the negative control (dead MSCs treated with methanol) showed 98 ± 2 of dead cells (positive for EthD-1). The gating of the FACS data for the 300 µM Bi$^{3+}$-labeled sample is shown in Figure 5.2. Gating of the data is crucial to obtain reliable results since the debris, and clusters of cells are excluded during this process, thus single MSCs can be analysed for fluorescence. Additionally, a known number of Bi$_4$C@US-tube-labeled MSCs and unlabeled MSCs (as controls) were collected to be digested and analysed by ICP-MS for bismuth.

Figure 5.1 – Viability of MSCs after being labeled with different concentrations of Bi$_4$C@US-tubes. Data were obtained by fluorescence-activated cell sorting (FACS) and are reported as mean ± SD.
Figure 5.2 – Representation of the gating for the cell viability data obtained by fluorescence-activated cell sorting (FACS) of unlabeled cells without dyes (unstained MSCs), positive control cells (unlabeled live MSCs), negative control cells (unlabeled dead MSCs), and Bi₄C@US-tube-labeled MSCs (300 µM Bi³⁺). (A) Gating of the single cells, (B) gating of the viable cells, and (C) diagram of the side-scattered light (SSC) and Allophycocyanin (ACP-A) of the viable cell population.

As shown in Figure 5.3, there is a direct relationship between the labeling concentration and the bismuth uptake. When the MSCs are labeled with concentrations from 10 to 80 µM, a minimal amount of bismuth uptake was achieved. However, a labeling concentrations higher than 100 µM, greater uptake of the Bi₄C@US-tubes material was
observed. Within the range of concentrations studied, the uptake varied from $1.2 \times 10^7$ to $1.6 \times 10^9$ ions of $\text{Bi}^{3+}$ per cell. The higher concentrations significantly exceeded our first generation material, Bi@US-tubes, which was reported to deliver $\sim 5 \times 10^7$ ions of $\text{Bi}^{3+}$ per cell using a labeling concentration of 24 μM $\text{Bi}^{3+}$.\(^8\)

![Graph showing uptake of Bi@US-tubes by MSCs](image)

**Figure 5.3** – Uptake of the Bi@US-tubes by MSCs, the concentration of $\text{Bi}^{3+}$ ion was obtained by ICP-MS. Data reported as mean value ± SD.

For further experiments, 300 μM $\text{Bi}^{3+}$ was used as the labeling concentration to obtain the greatest $\text{Bi}^{3+}$-ion loading within the MSCs, a dose that did not affected cell viability (~96% MSCs alive). TEM images of the labeled MSCs (Figure 5.4) showed that the Bi@US-tubes accumulated exclusively in the cytoplasm of the cells, with no evidence of translocation into the nucleus. The same result has been reported for similar materials, such as the Gadonanotubes,\(^10\) which are US-tubes loaded with Gd$^{3+}$-ion clusters and others analogous materials.\(^10,14\) However, the Bi@US-tubes are the first of a US-
tube-based material to become usually encapsulated within vesicles in the cytoplasm of the MSCs. The encapsulation of US-tube materials within vesicles has been previously observed for cancer cells (Hep3B and Hep G2), but never before reported for MSCs. In some of the vesicles, the characteristic formation of fiber-like structures that CNT materials often adopt in aqueous media is also clearly visible (Figure 5.4C). The fact that Bi$_4$C@US-tubes are seemingly being internalized by the MSCs via an active transport mechanism might be due to the surface charge of the nanomaterial. Bi$_4$C@US-tubes have a slightly more positive charge when compared to US-tubes and Gadonanotubes, as confirmed by its zeta potential (obtained using a Malvern Zen 3600 Zetasizer (Table 5.1). The present TEM data clearly demonstrates that Bi$_4$C@US-tubes do not cross the nuclear membrane, but instead, are only enclosed in vesicles which suggests an active cellular transport mechanism, as noted for other CNT-based materials used to label MSCs.\textsuperscript{205}
Figure 5.4 – TEM images of Bi₄C@US-tube-labeled cells. Yellow arrows indicate to the Bi₄C@US-tubes encapsulated in vesicles localized in the cytoplasm and red arrows point to the nucleous. (C) A enlarged image of the Bi₄C@US-tubes material, where fiber-like agglomerates can be seen.
Table 5.1 – Z-potential values for Pluronic® alone, SWCNTs, US-tubes, Gadonanotubes and Bi₄@US-tubes. *Values previously reported on Ref.²⁰⁴

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluronic* 0.17% (w/v)*</td>
<td>-19.3 ± 3.4</td>
</tr>
<tr>
<td>Full length SWCNTs*</td>
<td>-27.0 ± 1.7</td>
</tr>
<tr>
<td>US-tubes*</td>
<td>-53.8 ± 1.8</td>
</tr>
<tr>
<td>Gadonanotubes*</td>
<td>-44.3 ± 0.4</td>
</tr>
<tr>
<td>Bi₄C@US-tubes</td>
<td>-21.6 ± 2.3</td>
</tr>
</tbody>
</table>

5.3.3. Label retention of the Bi₄C@US-tubes material

In addition to studying the degree and mechanism of cellular uptake of Bi₄C@US-tubes, it is crucial to evaluate the potential clearance of the material from the MSCs. To determine this, the Bi³⁺-ion content in the culture medium was analyzed under two different experimental conditions to determine whether or not MSCs were actively releasing the intracellular Bi₄C@US-tubes material into the culture medium. In Group A, the culture medium was replaced every 24 h post labeling, while in Group B the culture medium remained unchanged. Figure 5.5 shows the Bi³⁺ ion concentration in the culture medium for the two groups. A gradual decrease of bismuth concentration was observed in the medium collected from cultures subjected to medium changes (Group A). Comparison between baseline (0 h) and the other time points values (24, 48 and 72 h) showed significant differences. For Group B, major release of the material occurred within the first 24 h, after which, the Bi³⁺-ion concentration in the culture medium remained nearly constant. The early release of bismuth in Group B may have resulted from cell death due to the initial
exposure to the Bi$_4$C@US-tube material, since it was found that ~4% of cells died after the labeling step (viability studies, Figure 5.1). Furthermore, the increase in the bismuth content could also be possibly due to the removal of residual material from the external cellular membrane. Since no major release of the material was observed over time in Group A (after 0 h), the same phenomenon of cell death and removal of bismuth from the extracellular matrix after initial labeling explains the results. In summary, this finding showed that the Bi$_4$C@US-tubes material remained mostly in the intracellular compartment of cells and a small fraction of the nanomaterial was cleared; similar findings have been reported previously for an analogue material.$^{10}$

![Figure 5.5 – Bi$^{3+}$-ion content in 3 mL of the culture medium after 24 h of labeling of MSCs with Bi$_4$C@US-tubes. Values reported in mean ± SD.](image)

To better understand the retention of Bi$_4$C@US-tubes within MSCs, the average Bi$^{3+}$-ion content within MSCs was obtained at 0 h and 72 h time points for both experimental groups. Labeled MSCs at baseline (0 h, 24 h post labeling with Bi$_4$C@US-
tubes) had $\sim 1.34 \times 10^9$ Bi$^{3+}$ ions/cell. At 72 h, the content for Group A and Group B were $4.10 \times 10^8$ Bi$^{3+}$ ions/cell and $1.06 \times 10^9$ Bi$^{3+}$ ions/cell, respectively. The significant decrease in the Bi$^{3+}$-ion content within MSCs for Group A could be due to dilution of the material due to cell division, which is expected to be more pronounced for Group A. Continuous refreshment of the culture medium has proven to enhance cell growth due to constant administration of nutrients. Nevertheless, by the end of the assay (72 h post-labeling), there was still a substantial concentration of bismuth within the MSCs for both experimental groups.

5.3.4. Growth kinetics

To study the effect of Bi$_4$C@US-tubes on the cell growth pattern and proliferation, a population doubling time (PDT) and colony-forming unit fibroblast (CFU-F) assays were performed, and Bi$_4$C@US-tubes-labeled MSCs were compared to control cells (unlabeled MSCs and cells treated with 0.17% Pluronic$^\text{®}$). The PDT assay provides quantitative information about how the cells are dividing over time. Figure 5.6 shows the growth curves for the three experimental variations of MSCs. As can be seen in the figure, no significant difference was found between the unlabeled MSCs, Pluronic$^\text{®}$-treated MSCs, and Bi$_4$C@US-tube-labeled MSCs. An initial decrease in the cell number from 24 to 48 h for all three conditions can be seen, which is expected to occur during the lag phase. The decline in cell number is more apparent for the Bi$_4$C@US-tube-labeled MSCs, and it could be attributed to cell death during the first 48 h. Nevertheless, the three tested cultures reached the same average number of cells by 144 h. A similar growth pattern was observed for Bi@US-tube-labeled MSCs (discussed in Chapter 3). The PDT values obtained using
Equation 3.1 are presented in Table 5.2. As seen in the table, it took about 22 h in average for the MSCs to doubled their cell number. No significant difference was observed among Bi₄C@US-tube-labeled MSCs and controls. These findings demonstrated that both CAs, Bi@US-tubes and Bi₄C@US-tubes exhibited no significant effect on growth of MSC cultures.

Figure 5.6 – The growth pattern for control unlabeled-MSCs, MSCs treated with Pluronic®, and Bi₄C@US-tube-labeled MSCs. Results showed no significant difference for the three cell culture conditions, mean ± SD, p > 0.05.
Table 5.2 – Population doubling time (PDT) values. Results reported as mean ± SD, p > 0.05.

<table>
<thead>
<tr>
<th>MSCs</th>
<th>hours</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>19.3 ± 3.1</td>
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<tr>
<td>Pluronic&lt;sup&gt;®&lt;/sup&gt;</td>
<td>24.8 ± 8.4</td>
</tr>
<tr>
<td>Bi&lt;sub&gt;4&lt;/sub&gt;C@US-tubes</td>
<td>20.1 ± 2.8</td>
</tr>
</tbody>
</table>

For the CFU-F, the colonies were counted after 2 weeks of plating the cells. The number of colonies for the Bi<sub>4</sub>C@US-tube-labeled MSCs were comparable to those of the unlabeled and Pluronic<sup>®</sup>-treated MSCs (Figure 5.7). Both treated cell cultures, the Bi<sub>4</sub>C@US-tube-labeled MSCs and the Pluronic<sup>®</sup>-treated MSCs, showed more colonies than control MSCs, although there is no significant difference in terms of CFU-F numbers (p = 0.34). These results shown that the ability of the MSCs to self-renew was not inhibited after treating the cells with Pluronic<sup>®</sup> and Bi<sub>4</sub>C@US-tubes.
5.3.5. MSCs differentiation

As multipotent progenitor cells, MSCs have the ability to differentiate into different cell types including progenitor cells for fat (adipocytes), bone (osteocytes) and cartilage (chondrocytes) tissue. This property of MSCs can be evaluated by promoting the MSCs to differentiate into other cell types in vitro upon appropriate exposure to differentiation-inducing media. As seen in Figure 5.8, Bi₄C@US-tube-labeled MSCs successfully showed evidence of intracellular lipid vacuoles (in red), calcium deposit (in red), and glycosaminoglycans activity (in blue), demonstrating adipo-, osteo-, and chondrogenic differentiation capabilities. For the chondrogenic differentiation assay, a cell sphere was kept suspended in differentiation media for 14 d. As seen in Figure 5.8C, the Bi₄C@US-tube material remained in the center of the labeled cell sphere, and direct prolonged-
exposure to the Bi$_4$C@US-tube material did not change the normal behavior of the MSCs. This confirms that the labeling of MSCs with the Bi$_4$C@US-tube material did not affect the differentiation of the MSCs, which suggests that Bi$_4$C@US-tube-labeled MSCs retain their therapeutic potential.
Figure 5.8 – Histochemical stains of unlabeled control and Bi₄C@US-tube-labeled MSCs after exposure to (A) adipogenic, (B) osteogenic, and (C) chondrogenic differentiation-induce media. In (A) the presence of lipid vacuoles appear in red as stained with Oil Red O; in (B), the presence of calcium was stained with Alizarin red, and in (C), Alcian blue stained the presence of glycosaminoglycans.

5.3.6. X-ray CT experiments for Bi₄C@US-tube-labeled MSCs

The potential of the Bi₄C@US-tube material to function as an intracellular contrast agent for X-ray CT was evaluated by preparing cell pellets of unlabeled MSCs and
Bi₄C@US-tube-labeled MSCs, as described above. Figure 5.9A shows Eppendorf tubes containing 200×10⁶ cells each. Unlabeled MSCs appear white, while labeled MSCs are dark due to cellular internalization of the Bi₄C@US-tube material. Analysis of regions of interest revealed HU values, which are an indicator of the ability of the material under study to attenuate X-rays with respect to water (0 HU) and air (-1000 HU). HU values obtained for the control (unlabeled) MSCs and for Bi₄C@US-tubes-labeled MSCs were 30 ± 9 and 214 ± 22, respectively. Thus, the HU value obtained for the Bi₄C@US-tube-labeled MSCs are approximately 2× greater than those previously reported for Bi@US-tube-labeled MSCs (110.1 ± 4.9 HU)

Figure 5.9 – (A) Photograph of the unlabeled MSCs (left) and Bi₄C@US-tubes labeled MSCs (right). (B) Conventional black and white CT image (coronal view) and (C) the CT colored image.
5.4. Conclusion & Future Directions

The work presented here and in Chapter 4 introduces X-ray CT as a new imaging technology to image MSCs. Many other imaging techniques have been investigated for this purpose, such as MRI and optical imaging modalities, however CT offers advantages over these imaging technologies such as fast data collection and high spatial resolution. The new CNT-based CA reported here, Bi$_4$C@US-tubes, is taken up by MSCs (without the use of transfection agents) via an active transport mechanism to form aggregates within vesicles in the cytoplasm of the cells. After internalization of the Bi$_4$C@US-tube material within the cells, the MSCs become opaque to X-rays, allowing CT images of the labeled MSCs to be brighter when compared to unlabeled control cells. The Bi$_4$C@US-tubes do not alter the viability, proliferation, or differentiation potential of the MSCs. This new intracellular CA material with 20% bismuth by weight is approximately 2× brighter when localized within MSCs than the first generation material, Bi@US-tubes, which contained only ~2.6% bismuth by weight.

Improvements are needed in order to obtain greater X-ray attenuations of labeled cells. Some likely strategies for improvement might include increasing the suspendability of the CNT material in aqueous media, thus increasing the Bi$^{3+}$-ion concentration in the labeling solution and possibly combining different metals of high atomic number in the same CNT platform to increase the X-ray attenuation. However, the present Bi$_4$C@US-tube formulation (and the Bi@US-tubes predecessor) presented in this work represent significant progress in the development and implementation of intracellular CNT-based CAs for X-ray-based imaging of live cells.
To the best of our knowledge, Bi@US-tubes and Bi₄C@US-tubes are the first materials to be used as intracellular CAs for the imaging of cells by X-ray CT. Other methods reported previously to track cells by CT, have not involve the CA being intracellular in nature. For example, alginate-poly-L-lysine-alginate microcapsules containing barium sulfate or bismuth sulfate have been investigated as radiopaque capsules to track human cadaveric islets by encapsulating the cells in the inner space of the capsules. Similar microcapsules containing gold nanoparticles and perfluorocarbons have been also reported for multimodal imaging, including CT, and for the encapsulation of different types of cells.

5.5. Comparing Bi@US-tubes and Bi₄C@US-tubes

In this Chapter, as well as in Chapters 3 and 4, two CNT-based materials containing bismuth are presented and discussed. Both compounds have been used to intracellularly label porcine MSCs to increase the X-ray attenuation of the cells, making them brighter when analyzed with X-ray-based imaging modalities. The first intracellular CA, Bi@US-tubes, contains bismuth ions within the cavities of the US-tubes, while Bi₄C@US-tubes is derived from US-tubes and Bi(III)-oxo salicylate clusters containing four bismuth atoms in its core. Both CAs were suspended in a 0.17% Pluronic® solution and added directly to cell culture flasks. No major differences between the two labeled cells; Bi@US-tube-labeled MSCs and Bi₄C@US-tube-labeled MSCs, were observed. However, a more extensive study was performed with the Bi₄C@US-tube material, therefore, additional experiments should be conducted with Bi@US-tubes in order to make a better comparison between the
two CAs. For example, 14 different labeling concentrations (from 10 to 300 μM Bi\(^{3+}\)) were evaluated for the Bi\(_4\)C@US-tube material while just one (24 μM Bi\(^{3+}\)) was used for Bi@US-tubes. High cell viabilities were achieved for both agents, however, the proliferation curves were slightly different. A higher average number of cells was observed for the Bi@US-tube-labeled MSCs throughout the proliferation assay compared to unlabeled control cells, suggesting that the Bi@US-tubes better contribute to the rapid growth of cells. Nevertheless, Bi\(_4\)C@US-tube-labeled MSCs showed a very similar growth curve to the one obtained for Pluronic®-treated MSCs.

Another aspect to consider is the cellular localization of the material and the mechanism of uptake. TEM data has shown that Bi\(_4\)C@US-tubes localized within vesicles in the cytoplasm of the MSCs, which indicates that an active transport mechanism takes place. Electron microscopy analysis of the Bi@US-tube-labeled MSC must be performed to get more information about the nature of this material, its interaction with MSCs, and how it compares to Bi\(_4\)C@US-tubes in term of incorporation into MSCs. However, it is expected that Bi@US-tubes localize in the cytoplasm of the cells, but not within vesicles. In a similar study, the Gadonanotubes, which are US-tubes loaded with Gd\(^{3+}\) ion clusters have also been shown to agglomerate in the cytoplasm of MSCs, but no evidence of vesicles encapsulating the material was found. Furthermore, neither of the two CAs altered the ability of the MSCs to differentiate into adipocytes, osteocytes, and chondrocytes. Table 5.3 summarizes some of the key data obtained in the previous Chapters.
Table 5.3 – Summary of the results for MSCs labeled with Bi@US-tubes and Bi₄C@US-tubes. * Value obtained from Ref. 8.

<table>
<thead>
<tr>
<th></th>
<th>Bi@US-tube-labeled MSCs</th>
<th>Bi₄C@US-tube-labeled MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling concentrations studied</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Labeling concentration used</td>
<td>24 μM Bi³⁺</td>
<td>300 μM Bi³⁺</td>
</tr>
<tr>
<td>Bi³⁺ ions/cell</td>
<td>5.0 × 10⁷*</td>
<td>1.6 × 10⁹</td>
</tr>
<tr>
<td>Viability</td>
<td>99.5 ± 0.3</td>
<td>97.4 ± 0.5</td>
</tr>
<tr>
<td>Proliferated?</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>PPT value (h)</td>
<td>19.9 ± 2.9</td>
<td>20.1 ± 2.9</td>
</tr>
<tr>
<td>Mechanism of uptake of the material</td>
<td>Unknown</td>
<td>Active</td>
</tr>
<tr>
<td>Adipogenic differentiation</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Osteogenic differentiation</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Chondrogenic differentiation</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>
Chapter 6

Encapsulation of doxorubicin in an FDA-approved iron oxide-polymer complex

6.1. Introduction

Doxorubicin (DOX) is one of the most common chemotherapy drugs used to treat a wide range of malignancies. However, its main drawback is the cardiotoxicity associated with it. In an effort to reduce DOX toxicity, the drug has been previously encapsulated in different materials to control its release, as well as to increase selectivity, thus avoiding its binding to proteins or other macromolecules. Many formulations containing superparamagnetic iron oxide nanoparticles (SPIONs) and DOX have been reported previously (refer to Section 2.2.3, page 19), however, most of these complexes are prepared by covalently binding DOX to a polymeric layer around the SPIONs or directly to the particles, which can alter the activity of the drug. This chapter presents an easy method to
encapsulate DOX in an ionic complex composed of two FDA-approved compounds, which makes this formulation promising for human use without the need of long screening processes and extensive pre-clinical trials. This nano-sized delivery system carries a therapeutic drug (DOX) and a diagnostic imaging agent (Feraheme®) with a targeting function. In order to achieve the association of DOX with Feraheme®, the drug was first bound to heparin, a polymer with a high negative charge. Detailed information regarding heparin and Feraheme® can be found in Sections 2.2.4 and 2.2.5 (page 21-22), respectively.

SPIONs such as Feraheme®, have drawn particular interest as a $T_2$-weighted CA for MRI and for their capability to be guided to a target site using strong external magnetic fields. Therefore, anticancer drugs such as DOX can be indirectly monitored by MRI and magnetically targeted when associated with such nanosystems. Additionally, DOX is inherently fluorescent which allows the drug to be track by confocal microscopy or by other fluorescence-based imaging modalities. In this way, the release of DOX from the nanocarrier can be monitored in vivo.

The nanocomplex described in this chapter is termed DOX-HFe, and it has the advantaged that it does not require a chemical modification of either DOX, heparin, or Feraheme®, thus, making its preparation easy and efficient. Furthermore, since DOX is not being chemically functionalized, its anticancer activity should be the same as the free drug.
6.2. Materials & Methods

6.2.1. Preparation of the DOX-heparin complex

All experiments were conducted in the dark. The DOX-heparin (DOX-H) complex was prepared immediately before use by dissolving 1 mg of DOX (Sigma Aldrich) in 0.44 mL of HPLC water, and 60 μL of heparin (1000 U/mL, Sagent Pharmaceuticals®) to achieve a final concentration of 2 mg/mL of DOX and 120 U/mL of heparin. The maximum degree of binding of DOX to heparin has been previously reported to be attained at these concentrations. The sample was subsequently centrifuged at 1200 rpm for 5 min. After centrifugation, the bright red pellet was concentrated at the bottom of the centrifuge tube containing the DOX-H complex (Figure 6.1). As a control, a solution of just DOX (2 mg/mL) was also centrifuged to determine if DOX precipitates under the same conditions.

![Figure 6.1 – DOX-H complex preparation.](image)

Aliquots of the initial solution (containing DOX and heparin) and of the supernatant obtained post- centrifugation were collected to calculate the encapsulation efficiency.
percent (EE %) using Equation 6.1. The concentration of DOX was determined by high-performance liquid chromatography (HPLC) analysis using fluorescence detection. An Acquity UPLC H-class System equipped with a fluorescent detector with excitation and emission wavelengths set at 440 nm and 585 nm, respectively, was used. The mobile phases were water and methanol, using a UPLC BEH C18 column. DOX concentrations were calculated with reference to a calibration curve.

\[
\text{[DOX]_{total}} - \text{[DOX]_{supernatant}} \times \frac{\text{[DOX]_{total}}}{100}
\]

Equation 6.1 – Encapsulation efficiency percent (EE %)

The remaining supernatant was removed and 500 µL of water was added, followed by centrifugation. The washing step was repeated at least 5 times to avoid free-DOX in the sample. Finally, all the liquid was carefully removed without disturbing the DOX-H complex, which appeared as a bright red solid pellet at the bottom of the centrifuge tube.

6.2.2. Preparation of the DOX-HFe complex

After preparing the supramolecular DOX-H complex, the second step involves the addition of Feraheme® to the mixture. To prepare the DOX-HFe complex, 15 µL (450 µg of elemental iron) of Feraheme® (ferumoxytol solution, AMAG Pharmaceuticals, Waltham, MO, USA) was added to the sample and mixed several times using a micropipette. The sample, which looks like a dark brown paste after the addition of the SPION, was lyophilized overnight. Finally, 500 µL of water was added and magnetic manipulation was utilized to attract the magnetic particles to the bottom of the centrifuge
tube (see Figure 6.2). Aliquots of the supernatant were collected for HPLC analysis and the EE % was also calculated for this step. The sample was resuspended in water and magnetic manipulation was performed followed by removal of supernatant. This process was repeated 8-10 times to avoid free DOX and free DOX-H in the sample. The resulting complex was resuspended in water and analyzed using a Shimadzu 2425 UV-visible spectrophotometer and a Horiba NanoLog Spectrofluorometer. The fluorescence was assessed using a 2 nm slit width from 495 nm to 800 nm with an excitation wavelength at 480 nm passing through a 2 nm slit width. Analysis of iron was conducted by ICP-OES to calculate the amount of DOX per SPION.

![Figure 6.2 – Preparation of the DOX-HFe complex, followed with magnetic manipulation to remove free-DOX from the sample.](image)

### 6.2.3. Interaction study

To better understand the interaction of DOX in the DOX-HFe complex, samples of DOX-HFe were exposed to NaCl\(_{(aq)}\) as an electrostatic shielding agent, and to ethanol as a hydrogen-bond disrupting agent. The Dox-HFe complex was suspended in 1.5 mL
Eppendorf tubes in different concentrations of NaCl\textsubscript{(aq)} or different v/v ratios of water/ethanol. After 10 min, a magnet was placed under the samples for ~5 min to bring the magnetic particles to the bottom of the tubes and aliquots of the supernatant were collected for HPLC analysis to determine the concentration of free-DOX in the samples.

6.2.4. Release study

To evaluate the possible kinetics of \textit{in vitro} release of DOX from the complex, aliquots of 0.5 mL of DOX-HFe were transferred into dialysis membrane cassettes (Slide-A-Lyzer\textsuperscript{®} Dialysis Cassette, cut off 10 kDa, 0.1 – 0.5 mL capacity). The cassettes were placed into vials containing 100 mL of either PBS (pH 7.4) or citrate-buffered saline (CBS, pH 5.5). The samples were kept under constant stirring and aliquots of 0.5 mL were collected for up to 96 h for HPLC analysis. A solution containing the DOX-H complex was also dialyzed under the same conditions as the control.

6.2.5. Agglomeration study

The size and degree of agglomeration of the DOX-HFe complex were investigated. The hydrodynamic diameter and zeta potential were obtained using a Malvern Zen 3600 Zetasizer. For these studies, the DOX-HFe complex was prepared in HPLC water as described above, as well as in HEPES buffer (pH 7.0 – 7.6) to investigate agglomerates of the complex in pure water versus in a more organic media. For the dynamic light scattering (DLS) and zeta potential studies, the DOX-HFe complex was suspended in either water or HEPES at very low concentrations.
6.3. Results & Discussion

6.3.1. Loading of DOX on the DOX-HFe complex

With the aim of tracking DOX by MRI while minimizing DOX toxicity by increasing the selectivity of the drug using magnetic targeting, a complex containing DOX and SPIONs was prepared. The nanocarrier contained DOX, heparin and ferumoytol, an FDA-approved SPION sold as Feraheme®. The preparation of the complex does not require any chemical functionalization, which might alter the activity of the drugs. Instead, a complex containing DOX and heparin (DOX-H) is first prepared, which forms due to supramolecular interactions, followed by the incorporation of Feraheme®. During the first step, a precipitate containing DOX and heparin is obtained post centrifugation. As a control, DOX was centrifuged under the same conditions to determine if it precipitates at that concentration. No precipitate of DOX was observed post centrifugation at a concentration of 2 mg/mL.

After the preparation of the DOX-HFe complex as described in Section 6.2.2, The SPIONs adopt a bright red color that can be observed in Figure 6.3, while “naked” Feraheme® appears as a light brown color. Magnetic targeting was applied to show the response of both samples to an external magnetic field when suspended in aqueous solvents.
Figure 6.3 – Photographs of solutions of Feraheme® (left) and the DOX-HFe complex (right). Magnetic manipulation is used to show the response of both samples to an external magnetic field.

NOTE: Samples do not contain the same concentration of SPIONs.

The absorbance and fluorescence of the complex were assessed to confirm the presence of DOX in the formulation and to compared them to those of the free-DOX. Neither the absorbance or fluorescence intensities were used to determine DOX concentration. Figure 6.4 shows the absorption spectrum of a solution of DOX in water and the fluorescence spectrum of DOX in aqueous environments, including water, citrate-buffered saline and cell culture media. The fluorescence spectrum for DOX was collected in different solutions to determine if biological relevant media affect the inherent fluorescence of DOX. Figure 6.4 also shows the absorbance and fluorescence spectra for the DOX-HFe complex suspended in water. As seen in the figure, the absorbance spectrum for the DOX-HFe complex is slightly different from that of free DOX, however no
significant shift in the \( \lambda_{\text{max}} \) was observed. The fluorescence spectrum for both free DOX and DOX-HFe are almost identical, and no shift of the \( \lambda_{\text{max}} \) was observed.

Figure 6.4 – (A) UV/vis spectrum of a solution of DOX in water and (B) shows the fluorescence spectra of DOX in different biological mediums. (C) shows the absorbance for the DOX-HFe complex and (D) the fluorescence spectrum of the DOX-HFe complex in water.

NOTE: DOX concentration varies for all the samples presented in this figure.

HPLC equipped with a fluorescent detector was utilized to determine the concentration of DOX in the DOX-HFe complex by analyzing the initial and final concentrations of free DOX during the production of the nanocarrier. As an example, Table
6.1 shows the results for one of the samples and the EE % obtained for each step, calculated using Equation 6.1. On average, the EE % for the preparation of the DOX-H and DOX-HFe complexes were (85 ± 3)% and (98.0 ± 0.5)% respectively (reported in mean ± SD). The fact that the EE % is higher for the second step indicates that the addition of Feraheme® to the mixture does not disturbs the interaction between DOX and heparin, thus, very little release of DOX takes place.

The iron concentration of the DOX-HFe complex was obtained by ICP-OES and the number of particles in the sample was calculated using the following chemical formula: Fe5874 O8752 C11719 H18682 O9933 Na414.184,185 By obtaining the concentration of DOX and iron in the DOX-HFe complex it is possible to calculate the amount of DOX per iron content. About 14.9 mg DOX/mg Fe can be achieved when the DOX-HFe complex is prepared as described above.

Table 6.1 – Example of the concentrations of DOX obtained for each step of the DOX-HFe preparation and the encapsulation efficiency percent (EE %) obtained.

<table>
<thead>
<tr>
<th>I. DOX-H complex</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>[DOX]_{initial} (mg/mL)</td>
<td>[DOX]_{supernatant} (mg/mL)</td>
<td>EE %</td>
<td>[DOX]_{final} (mg/mL)</td>
</tr>
<tr>
<td>1.967</td>
<td>0.340</td>
<td>82.7</td>
<td>1.627</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. DOX-HFe complex</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>[DOX]_{initial} (mg/mL)</td>
<td>[DOX]_{supernatant} (mg/mL)</td>
<td>EE %</td>
<td>[DOX]_{final} (mg/mL)</td>
</tr>
<tr>
<td>1.627</td>
<td>0.0026</td>
<td>98.4</td>
<td>1.601</td>
</tr>
</tbody>
</table>
6.3.2. Interaction study

To evaluate which supramolecular forces are involved in the binding between DOX and the magnetic nanocarrier, an interaction study was performed. The DOX-HFe complex was exposed to different concentrations of NaCl$_{aq}$, for the disruption of ionic interactions, and to solutions of different ratios of water and EtOH, for the disruption of hydrogen bonds. Figure 6.5 shows the release of DOX from the magnetic nanocarrier. The concentration of DOX was determined by HPLC and iron concentration was obtained by ICP-OES. As seen in the figure, there is a significant release of DOX from the nanocarrier when exposed to ionic solutions, as well as when it is suspended in a hydrogen-bond disrupting agent, indicating that DOX and the nanocarrier are linked by ionic interactions and by hydrogen bonds. However, the results show that a greater release of DOX is obtained when the ionic interactions of the complex are challenged. DOX is positively charged (one protonated amine) when dissolved in aqueous solutions, while heparin is highly negative due to the sulfate groups along the polymeric chain, thus molecules of DOX can easily interact with the sulfate groups to create an ionic complex.
6.3.3. Release study

To study the possible *in vitro* release of the drug in different environments, the DOX-HFe complex was dialyzed in two aqueous solutions with different acidities. The dialysis cassettes were submerged in PBS (pH 7.4) or CBS (pH 5.5). As a control, a solution containing the DOX-H complex was also dialyzed in the two buffers. As seen in Figure 6.6, the release of DOX increased with decreasing pH values, showing the acid-sensitive nature of the DOX-H and DOX-HFe complexes, however, there are differences between their release patterns. When the complexes are exposed to neutral pH, there is a slower release of DOX from the DOX-HFe complex than from the DOX-H complex. This is preferable since the release of DOX at normal pH could cause high concentrations of DOX in healthy tissue, thus, leading to toxicity. The fact that DOX releases more slowly when it is associated with Feraheme® indicates a stronger interaction of DOX in the DOX-HFe carrier that in the DOX-H complex. However, the DOX-HFe complex shows a more rapid
response to acidic conditions when compared to DOX-H. The greater release of DOX was attained with DOX-HFe at acidic pH, with a ~ 35% of DOX being released in 96 h. A very similar release pattern was reported previously by an ionic complex composed of DOX and γ-polyglutamic acid. It is crucial to understand the release of DOX to determine the administration doses of the formulation and how often it must be administrated. Furthermore, the slow release of DOX allows a window time for the nanoparticle to reach the tumor sites (by magnetic targeting), before releasing the drug.

![Graph showing DOX release from DOX-H and DOX-HFe complexes in PBS and CBS over time.](image)

**Figure 6.6 – Release of DOX from the DOX-H and DOX-HFe complexes in phosphate-buffered saline (PBS) and citrate-buffered saline (CBS).**

### 6.3.4. Agglomeration study

According to the Fereheme® manufacturer, the SPIONs have a diameter of about 20 – 30 nm. However, the diameter of the DOX-HFe complex might differ from this, especially if it forms aggregates due to the addition of the heparin coating. Thus, the size
of the complex was analyzed by DLS in both water and HEPES, which is an organic chemical buffering agent with a pH of 7.0 – 7.6. The chemical structure of HEPES is shown in Figure 6.7. The neutral pH of the solvent is crucial since acidic conditions cause the prompt release of DOX, as demonstrated above.

![Chemical structure of HEPES](image)

**Figure 6.7 – Chemical structure for HEPES, an organic chemical buffering agent (pH 7.0 – 7.6).**

The hydrodynamic size of the DOX-HFe complex in both water and HEPES buffer was attained and compared to that of Feraheme®. As seen in Figure 6.8, the DLS data shows that when the magnetic nanocarrier is suspended in water, relatively big agglomerates are formed. For the DLS data acquisition, the samples were diluted in water to obtain very low concentrations of the material, including the DOX-HFe suspension in HEPES. Therefore, even when the DOX-HFe complex is dispersed in a diluted solution of HEPES, only single particles are obtained. The zeta potential of the particles was also measured and it seemed to correlates with the particles size. Table 6.2 shows the zeta potential for Feraheme® in water and for the DOX-HFe complex in water and HEPES buffer. A slightly more cationic value is attained when water is used as the solvent for the DOX-HFe complex. However, more experiments are needed to better understand the ionic
interaction of the material when suspended in HEPES in order to analyze in details the differences in the zeta potential among the samples.

Furthermore, the concentration of DOX in the DOX-HFe complex was determined when using HEPES buffer as the solvent and no significant difference in the DOX concentration was found. HEPES buffer has a neutral pH (7.0 – 7.6), therefore it should not contribute to the rapid release of DOX from the nanocarrier. Likewise, the buffer solution is highly diluted in water, thus, its function as a hydrogen-bond disrupting agent is negligible.
Figure 6.8 – DLS data for (A) Feraheme® in water, DOX-HFe complex in water and DOX-HFe complex in HEPES buffer.
Table 6.2 – Zeta potential of Feraheme® in water and the DOX-HFe complex in both water and HEPES buffer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Z potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feraheme®</td>
<td>-18.0 ± 2.8</td>
</tr>
<tr>
<td>DOX-HFe in water</td>
<td>-13.9 ± 1.4</td>
</tr>
<tr>
<td>DOX-HFe in HEPES</td>
<td>-18.8 ± 0.4</td>
</tr>
</tbody>
</table>

6.4. Conclusion & Future Directions

This chapter describes the easy preparation of a new magnetic nanocarrier termed DOX-HFe, which contains only FDA-approved drugs. Beside carrying an anticancer drug, DOX-HFe can be tracked in vitro and in vivo by fluorescence-based imaging modalities and MRI. The complex is derived from DOX, heparin and Feraheme®, and the three components interact with one another by supramolecular forces, mainly by ionic interactions as showed in an interaction study where the ionic- and hydrogen-bonds of the complex were challenged. Since no chemical functionalization of any kind is carried out for the preparation of the complex, the activity of the drugs should not be compromised. Furthermore, it was demonstrated that a more rapid release of DOX, from the magnetic nanocarrier, is obtained when it is suspended in acidic solutions. Such response is desirable for therapies targeted to treat tumors since it has been proven that tumors have a more acidic environment compared to healthy tissues. Thus, the pH-controlled release of the drug is expected to significantly contribute to the therapeutic profile of the complex and minimize the toxicity associated with DOX.
Furthermore, the degree of agglomeration of the DOX-HFe complex can be controlled according to the solvent in which the formulation is prepared. As represented in Figure 6.9, the DOX-HFe complex forms agglomerates when prepared in HPLC water. However, single particles coated with heparin-DOX were attained when the formulation is prepared in HEPES buffer. More experiments must be performed to further understand the chemical behavior and differences between agglomerates and single particles. Similarly, *in vivo* experiments should be conducted to investigate which form, aggregated or single particles, provides the best circulation time, infiltration within tumors, and uptake within cancer cells. Macrophages tend to uptake nanoparticles according to their size, coating, charge, and other properties. It will be essential to investigate the degree of uptake by macrophages, if any, when single particles versus agglomerates are administrated.

![Figure 6.9 – Schematic of the behavior of DOX-HFe in water and in HEPES buffer. When suspended in water, the DOX-HFe complex form relatively large agglomerates that are not present when the complex is suspended in HEPES buffer.](image)

The DOX-HFe complex was produced with the objective of administrating the agent i.v. while strong external magnetic fields are used to direct the complex to tumor
sites in order to increase tumor selectivity. Another alternative is the loading of stem cells with the DOX-HFe complex to double the targeting feature. Mesenchymal stem cells are known to exhibit tropism for tumor microenvironments. By internally loading stem cells with DOX-HFe, the cells will work as capsules to deliver the drug to tumor sites, increasing selectivity. This particular approach is critically important to consider when tumors are located in areas where magnetic fields cannot be easily reach. Additionally, when external magnetic fields are applied, it might be easier to manipulate cells loaded with DOX-HFe than the single particles itself due to a higher number of magnetic domains working together when the nanoparticles are enclosed in “packages”. More importantly, it will be essential to evaluate which approach, if the free formulation (administered i.v.) or the DOX-HFe complex loaded within stem cells, will work better in terms of drug delivery, efficacy treating the malignancy, and decrease in toxicity and side effects.
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