Scalable Magnetically Enhanced Transport Mechanisms of Living Microrobots for Cancer Therapy

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Scalable magnetically enhanced transport mechanisms of living microrobots for cancer therapy

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To the women of Iran…
Acknowledgment

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Abstract

Despite advances in nanomedicine and immunotherapy that have led to substantial improvements in cancer therapy, effective drug delivery remains a major hurdle for successful long-term treatment. Cell-based delivery systems that exhibit tumor tropism like bacteria have been proposed as therapeutic agents capable of addressing persisting challenges such as off-target effects and insufficient drug distribution inside the tumor. The benefits offered by the intrinsic properties of these living therapeutics can be further enhanced by application of external forces. The human body is transparent to magnetic fields, making this external source of energy an especially promising means for remote manipulation of the cell-based systems in vivo. Intrinsically magnetically responsive bacteria, also referred as magnetotactic bacteria (MTB), present a unique opportunity for combining both innate and external tumor targeting strategies within a single drug delivery platform.

This thesis investigates magnetically assisted transport mechanisms to improve tumor drug delivery by using MTB as a model organism. Rotating magnetic fields (RMF) are chosen as the primary stimulus for magnetic actuation due to the scalability of magnetic torque-based techniques for clinical applications. Starting with remote micro manipulation of bacteria, ferrohydrodynamic phenomena associated with dense suspensions of MTB are studied experimentally and computationally, conceptualizing them as a living ferrofluid. Benchmarking against a synthetic ferrofluid composed of a suspension of iron oxide nanoparticles (IONPs) revealed that the MTB suspensions exhibit an increase of more than two orders of magnitude in flow generated per gram of magnetic material. Detailed comparison of the MTB and IONPs further support the use of bacteria as efficient flow mediators, converting the magnetic energy into a more homogeneous torque-driven fluid motion.

After identifying MTB as promising torque actuators, the torque-driven transport mechanisms behind their capacity to overcome biological barriers are elucidated through the establishment of computational and in vitro models. Enhanced surface exploration is shown to increase the likelihood of translocation in the presence of dynamic gaps observed in physiological barriers. Microfluidic devices incorporating collagen gels and endothelialized channels were fabricated as physiologically relevant models for identifying suitable actuation parameters. In agreement with the results of these test platforms, a subsequent in vivo study demonstrates enhanced delivery of MTB through actuation with RMF.
As a crucial step towards targeted actuation at larger scales, a scheme for spatially selective manipulation of MTB is identified based on application of a static gating field to suppress the magnetic torque in off-target areas. The application of a selection field at small scales is shown to localize the influence of actuation to the target, leaving off-target areas nearly unaffected. Lastly, the scalability of this actuation scheme is demonstrated by steps taken to design and build a mouse scale setup for in vivo studies. The multi-component setup can generate an RMF of up to 20 mT and a field free region (FFR) with an average 1 cm resolution that can be moved in space by addition of the offset fields.

The thesis concludes with a summary and remarks on future in vivo studies enabled by the development of a mouse scale setup. Potential extensions of the findings to other magnetically responsive cell-based systems are also discussed.
Zusammenfassung

Dank Fortschritten in der Nanomedizin und Immuntherapie kam es zu erheblichen Verbesserungen in der Krebstherapie, jedoch stellt die wirksame Arzneimittelabgabe nach wie vor eine große Hürde für eine erfolgreiche Langzeitbehandlung dar. Zellbasierte Systeme, wie z.B. Bakterien, die Tumortropismus aufweisen, wurden als Therapeutika vorgeschlagen, da sie möglicherweise in der Lage sind anhaltende Herausforderungen wie Off-Target-Effekte und unzureichende Arzneimittelverteilung im Tumor anzugehen. Die Vorteile, die durch die intrinsischen Eigenschaften dieser lebenden Therapeutika geboten werden, könnten durch Anwendung externer Kräfte weiter verbessert werden sowie auch deren Sicherheit erhöht werden. Der menschliche Körper ist für Magnetfelder transparent, was diese externe Energiequelle zu einem besonders vielversprechenden Mittel zur Fernmanipulation zellbasierte Systeme macht. Intrinsisch magnetische Bakterien, auch bekannt als magnetotaktische Bakterien (MTB), bieten eine einzigartige Gelegenheit, um sowohl natürliche als auch externe Strategien der gezielten Krebstherapie innerhalb einer einzigen Plattform zur Arzneimittelabgabe zu kombinieren.


Nachdem MTB als vielversprechende Aktuatoren für Drehmoment-basierten Transport identifiziert wurden, werden die Mechanismen, die ihrer Fähigkeit biologische Barrieren zu überwinden zu Grunde liegen, mittels Computer- und In-vitro-Modellen untersucht. In

Als entscheidender Schritt hin zu einer gezielten Aktuierung in größerem Maßstab wird ein Schema zur räumlich selektiven Manipulation von MTB identifiziert, das auf der Anwendung eines statischen Gating-Felds basiert, um das magnetische Drehmoment in Bereichen ausserhalb der Zielfläche zu unterdrücken. Es wird gezeigt, dass die Anwendung eines Selektionsfeldes den Einfluss der magnetischen Aktuierung auf das Ziel lokalisiert, wobei Off-Target-Bereiche nahezu unbeeinflusst bleiben. Schließlich wird die Skalierbarkeit dieses Aktuierungsschemas demonstriert, indem das Design und die Umsetzung eines Systems für In-vivo-Studien in Mäusen beschrieben wird. Der Multikomponentenaufbau kann ein RMF von bis zu 20 mT und eine feldfreie Region (FFR) mit einer durchschnittlichen Auflösung von 1 cm erzeugen, die durch Hinzufügen der Offset-Felder im Raum verschoben werden kann.

Die Dissertation schließt mit einer Zusammenfassung und Anmerkungen zu zukünftigen In-vivo-Studien, die durch die Entwicklung dieses Systems ermöglicht werden. Mögliche Erweiterungen der Ergebnisse auf andere magnetisch ansprechende zellbasierte Systeme werden ebenfalls diskutiert.
List of Publications

Publications that contributed to this thesis, ordered according to their appearance in subsequent chapters:


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* Co-first authors
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1 Introduction and background

Sections 1.2 and 1.3 of this chapter are adapted from (1). The author acknowledges the contributions from Tinotenda Gwisai as the co-first author of this publication.

1.1 Drug delivery in cancer

Cancer continues to evade treatments in many cases, causing nearly 10 million deaths worldwide in 2020 (2). It can be understood as a multistep process starting with mutations that render cells selfish members of a multicellular community and culminates in the formation of malignant tumors (3). Well-characterized hallmarks include uncontrolled proliferation and evasion of normal programmed cell death, as well as the ability to ignore growth suppressors (4, 5). Furthermore, cancer cells are known to induce angiogenesis and acquire invasiveness (4, 5), and the tumors they form are is able to establish a favorable tumor microenvironment (TME) by recruiting stromal cells (6).

In the battle against this disease, the goal is to eradicate all of these malignant cells while ensuring that the overall health of the host organism is not jeopardized. Selectivity is key—yet today’s treatment modalities only partially satisfy this need and typically require accepting trade-offs between efficacy and off target effects. Chemotherapy is targeted towards rapidly dividing cells, and surgery, if possible, relies on spatial control of the intervention (7). Radiotherapy also partially features both of these strategies for selectivity (7). However, the extent of selectivity offered by these conventional treatments is far from ideal, and depending on location, type, and stage of the cancer, different combinations may be used, resulting in a variety of side effects (8, 9).

Nanomedicine was first introduced in the 1960s and presented the concept of nanoscale drug carriers that could tailor the ultimate effect of the drug (10). Higher control over drug release through custom-designed nanocarriers potentially offers substantial benefits for drugs that suffer from poor bioavailability, short plasma half-life, or narrow therapeutic index (10–12). The expectations of nanomedicine were raised even further after the discovery of the
enhanced permeability and retention (EPR) effect in 1986 (13, 14). The EPR effect posits that nanoparticles (NPs) and macromolecules preferentially accumulate in tumors due to the presence of larger gaps in endothelial lining of the capillaries in tumor tissue (15, 16). This is accompanied by rising concentration of these nano-sized agents inside the tumor over time in the absence of functional lymphatics where the tissue “retains” the drug (17).

Nanotechnology in medicine enabled tangible advances towards the ultimate goal of therapies where many NP-based formulations were approved by Food and Drug Administration (FDA) (12). Doxil, liposomal doxorubicin coated with polyethylene glycol (PEG), and Abraxane, paclitaxel bound to albumin, are two examples that have provided substantial survival benefits clinically (18). However, limited delivery efficiency and the translational gap between preclinical models and human patients have drastically lowered the expectations of achievable therapeutic outcomes (19).

A meta-analysis on the delivery efficiency of the NPs highlighted the indispensable need for strategies to improve this crucial aspect of the treatment (20). Various custom-tailored characteristics such as size, shape or material hardly indicated any difference in terms of enhanced transport under specific conditions. Roughly 1% efficiency was estimated which implies complications in translating the method as it requires high dosage. This bottleneck is a result of biological and physical hindrances in the systemic delivery (19, 21), detailed in the following.
Various classifications of the published studies on NP delivery into tumors reveal low delivery efficiency without significant trend over time and among different NP and tumor characteristics. Reproduced from (20).

1.1.1 Biological barriers in systemic delivery

Poor delivery efficiency in cancer is attributed to the physical and biological barriers that a drug alone or with its carrier faces along its journey to reach the target (22, 23). These barriers are heterogenous across patients and are present at different levels ranging from organ to subcellular scale (19, 24). The focus is here is placed on drugs that are introduced intravenously. However, it should be noted that also upon administration through other routes, distribution of the drug eventually takes place through blood circulation (25, 26). It takes few minutes for drugs to widely spread in the circulation, and mononuclear phagocytic system (MPS) along with renal system form the first barrier following the systemic administration (27). MPS consists of family of cells with phagocytic activity distributed throughout the body.
Among them, monocytes in blood, Kupffer cells in liver, macrophages of spleen and dendritic cells are the most relevant cell types in the context of drug delivery. A major part of the injected dose of delivery systems is taken up by these cells and accumulate in liver and spleen \((20, 28)\). Drugs and carriers smaller than 5 nm undergo renal clearance and are excreted from the body in urine \((18, 21)\). These mechanisms, together with mixing in the whole blood, lead to nonspecific distribution of the drug. Within the circulation, blood flow and shear stress also act as physical barriers for delivery systems to reach their targets. Shear forces may disintegrate different components of a delivery system and fluid flow can interfere with extravasation at the target site \((29–31)\).

The next group of barriers is present locally at the level of the tumor tissue. Cancer cells with acquired self-sufficiency in signaling growth and proliferation demand high levels of oxygen and nutrients \((32)\). One key mechanism to meet this demand is angiogenesis, i.e. sprouting of new capillary vessels from pre-existing ones, which is mainly triggered by production of vascular endothelial growth factor (VEGF) from the cancer cells \((33)\). Ultimately, the high rate of proliferation in endothelial cells results in the formation of highly abnormal and immature blood vessels. The tumor vascular network is described as comparatively chaotic and disorganized with increased tortuosity \((34)\). Blood vessels are hyperpermeable due to incomplete formation of tight junctions. Absence of pericytes and smooth muscle cells in addition to defected basement membrane also contribute to this leakiness \((35)\). These characteristics have motivated the development of several types of therapies such as NP-based drug formulations that rely on the EPR effect. However, the effect has been reported to be inconsistent in human patients \((36)\), and other extravasation mechanisms were also proposed recently for NPs \((37)\). Compressive forces driven by uncontrolled proliferation of the cancer cells increase functional and structural abnormalities of blood vessels and cause malfunctioning of the lymphatic vessels \((15, 32)\).

Leaky vasculature and impaired lymphatic function cause the accumulation of fluid in the extravascular region, culminating in elevated interstitial fluid pressure (IFP) in tumors \((33)\). Furthermore, tortuous and compressed blood vessels are associated with higher flow resistance leading to hypoperfusion of the tissue \((38)\). This characteristic, together with uneven distribution of the vessels which are mostly sparser at the core, gives rise to insufficient oxygen supply and hypoxic nodules in acidic environments \((39, 40)\). As a result, these irregularities
mostly act as barriers to sufficient drug transport by constraining the tissue perfusion, limiting access to certain regions of the tumor mass, and leaving diffusion as the only means of transport in the TME. In addition, hypoxic areas are hard to access for systemically administered drugs and less prone to be affected by radiotherapy, which make them responsible for cancer relapse following initial treatments (41, 42). Other characteristics such as reduced immune surveillance and development of a more drug resistance phenotype are also attributed to hypoxia (43).

When a delivery system crosses the endothelial barrier, it faces both stromal cells such as fibroblasts and cancer cells embedded in the extracellular matrix (ECM) which is comprised of components like collagen, fibronectin, and hyaluronan (20, 32). Most solid tumors are reported to show higher stiffness mainly caused by a dense ECM due to an increased amount of ECM fibers (44). Cancer-associated fibroblasts contribute to this phenomenon by synthesizing the ECM components in addition to their role in coordinating the invasion of the cancer cells (45, 46). Rapid growth of the cancer cells in a confined area also generates a solid stress and narrows interstitial pathways (47). These act as physical barriers limiting penetration of the delivery systems deep into the tissues where interstitial fluid flow is also impaired due to relatively high and uniform IFP (23). The nature of IFP distribution in certain tumors can even cause the fluid to carry away the drug towards the periphery. This special type of interstitial flow is known to induce lymphogenesis which is linked to metastatic behavior of the cancer cells (48). Nonspecific adhesion to ECM components presents another type of barrier at the same level (20, 25).

The last group of barriers are present when a drug or delivery system reaches the target cells. The cell membrane as the first layer consists of negatively charged phospholipid bilayer incorporating many transmembrane proteins heterogeneously located in its structure (19). As a result, negatively charged macromolecules or delivery platforms may experience a repulsive force at this level (49). In case of endocytosis by the cells, i.e. uptake via surrounding the material with a small portion of the cell membrane forming endosomes, later occurring physiochemical changes in lysosomes can change the fate of the internalized drugs/carriers through degradation (50). High ionic strength and low pH are mentioned as the main characteristics of this environment. While in the cytosol, the therapeutic outcome is still compromised by the action of multidrug efflux pumps (27). Overexpression of P-glycoprotein,
a member of ATP-binding cassette superfamily, is mainly responsible for this behavior in cancer cells (51).

Figure 1-2: Physical and biological barriers in drug delivery to solid tumors. A) Free drugs and delivery systems encounter many barriers at different levels when administered systemically. Reproduced from (8) B) Detailed barriers at the tissue level caused by abnormal tumor microenvironment. Reproduced from (52).

These multi-level barriers create a complex journey for active pharmaceutical ingredients, whether as a free drug or in the form of a delivery system, to have the desired
therapeutic impact. Any proposed solution against cancer is doomed to fail without taking these drug delivery barriers into account. In this work, we focus mostly on the barriers at the tissue level and propose solutions for addressing complications associated with drug delivery at this scale. Therefore, a better understanding of the physical limitations of drug transport is a crucial step before introducing delivery systems capable of circumventing some of the mentioned barriers.

1.1.2 Physical limitations in tumor drug transport

In order for systemically injected drugs to achieve the highest efficacy in solid tumors, it is vital that they distribute uniformly across the tissue \((17, 23, 53)\). Failure in accessing all cancer cells gives rise to tumor relapse after a temporary inhibition of growth. As a result, knowing transport mechanisms associated with the delivery of drugs into the tissue enables development of delivery systems suited to the TME \((54)\). As a drug, whether a molecular compound or a NP-based formulation, travels through the bloodstream towards its target, multiple stages with varying dominant physical principles are encountered \((25)\).

General mass transport processes are described by the convection-diffusion equation \((55)\), which states that the time change of a quantity \(C\) at any point is equal to the net transport of that quantity plus the rate \(R\) at which it is generated or removed:

\[
\frac{\partial C}{\partial t} = \nabla \cdot (D\nabla C - \nu C) + R \quad 1-1
\]

Net transport is defined as divergence of summation of the diffusive flux, \(D\nabla C\), and the convective flux, \(\nu C\), where \(D\) is the diffusion coefficient and \(\nu\) represents the fluid velocity vector. The convective term acting like the “public transport” in fluid mechanics by carrying the species (drugs) over relatively large distances (different organs and tissues inside the body) \((25)\). Hence, this mode of transport is available wherever fluid pathways (blood vessels) are established. The second mass transport mechanism that plays role in drug delivery is molecular diffusion. This mode arises from the Brownian motion of molecules and small particles (drugs) resulting in a net transfer in the direction of the concentration gradient (from blood vessels to the surrounding tissue) \((56)\).

Comparing the time scales associated with each mode of transfer, one can derive the parameter describing the relative contribution of these two modes the drug transport:
Where \( L \) and \( U \) represent the relevant length and velocity scales, respectively. The Peclet number \( Pe \) reveals the dominant transport mechanism under different conditions as the function of the fluid velocity and size of the drug which in turn determines the diffusive flux (55, 57). Although nanoparticle transport within the body heavily relies on the convective contribution, this transport mechanism is typically disturbed in the tumor tissue due to the lack of proper interstitial flow (58). High interstitial fluid pressure, dense ECM, and hypoperfusion contribute to this effect (32, 59).

![Figure 1-3: Dominant mode of transport for delivery of different molecular compounds and NPs from blood flow to the tissue. Depending on the Stokes–Einstein radius, diffusion or convection can dominate the mass transport at different sites within the body. Reproduced from (25).](image)

Lastly, to better understand the details of local solute or particle transport at the tissue level, it is necessary to dissect each transfer mode within this complex microenvironment. Both diffusion and convection can divided into transvascular and interstitial components (60). Dependence of transvascular convection on hydraulic conductivity of the vessel wall highlights the role of pores, whether static or dynamic, and size of the therapeutic agent (61–63). Despite
the presence of the EPR effect in solid tumors, i.e. higher $L_p$, low transmural pressure renders this component negligible, as discussed above (32). The hydraulic conductivity $K$ of a neoplastic tissue, which enables modeling of the tissue as porous material, is also decreased compared to normal tissue (64). Dense ECM and rapid proliferation of cancer cells cause this drop by reducing the effective area of fluid pathways and increasing their tortuosity (25).

For transvascular diffusion, since it can occur through both transcellular and paracellular pathways, the vascular permeability is also influenced by other drug properties such as charge and lipophilicity (65). Large and/or hydrophilic drugs rely only on the paracellular pathways for transvascular transport. Here, the EPR effect confers specificity to cancerous tissues for large biomolecules and nanoparticles. Interstitial diffusion is also affected in nearly the same way as interstitial convection where added tortuosity and longer diffusion distances lead to drop in the equivalent diffusion coefficient in such porous media (66–68). However, lipophilic compounds can easily diffuse through the cell membranes which makes them less affected by these changes in the TME.

![Figure 1-4: Components of biological mass transfer across the endothelium and through the extravascular space. Tumor microenvironment generally exhibits higher vascular permeability and hydraulic conductivity than normal tissue due to relatively large intercellular gaps. Diffusion coefficient inside the tumor interstitium and its hydraulic conductivity are reduced because of dense ECM and uncontrolled growth of the cancer cells. Reproduced from (32).](image-url)
Another important parameter that can be derived from the convection diffusion equation is the penetration depth of the drugs into tissues. This distance is set by the relative contributions from the net transport flux and rate of uptake/removal from the medium. Various scenarios occur depending on the uptake kinetics of the drug and its size (25). The maximum extravascular tissue length that oxygen can diffuse into is around 100-200 μm (25, 69, 70). This limitation is the main cause for the formation of hypoxic and necrotic cells in tumors with abnormal vasculature. Assuming long-term systemic bioavailability, this distance was estimated to be approximately 85 μm for the low molecular weight anticancer drug doxorubicin (25). It should be noted that for drugs with relatively large diffusive time scale like nanoparticles, this rate imposes another limit for the maximum penetration depth. When the concentration of the drug starts to drop off in the blood, the spatial distribution of the concentration gradient changes in the tumor, halting further progression within the tissue. It should be noted that binding to the ECM network or clearance by the tissue-resident macrophages can also be regarded as uptake that limits deeper penetration of the drug.

Considering these physical principles governing the drug delivery from the transport perspective, we envisioned two general strategies to address the discussed challenges to selective delivery of intravenously introduced therapeutic agents. One strategy to improve the drug delivery into cancerous tissue is to recover the convective mode of mass transfer through local alteration of the fluid flow assisted by external energies. The second strategy involves exploiting living microrobots amenable to external control, capable of self-propulsion and responsive to the cues in the TME to reach poorly accessible cancer cells deep inside the tissue.

1.2 Active transport for enhanced tumor targeting

To effectively navigate through the TME and cross the biological barriers, active strategies have been proposed to circumvent the physical limitations associated with the physiological state of the drug transport. Microorganisms including cells and bacteria have naturally evolved different modes of motion to move through challenging environments (71–73). The bacterial flagellar motor is a fascinating rotary machine fueled by the hydrolysis of ATP (74). These powerful micromachines have been harnessed in biohybrid microrobot designs where bacteria as delivery shuttles are conjugated with synthetic materials. Physical stimuli including, light, acoustic waves, and magnetic fields have been applied to control the motion of
these biohybrid systems (75, 76). Leveraging innate motility of bacteria and external energies for tumor targeting may help to overcome the limitations and challenges encountered in the transport of non-living delivery systems. Overall, in light of the rise of living therapeutics, including mammalian and bacterial cells, strategies for external manipulation will become increasingly important (77–79).

1.2.1 Bacteria as living microrobots with tumor selectivity

A unique trait of bacteria that distinguishes them from other living cell-based therapeutics is their ability to swim in complex biological fluids, facilitated by the combination of onboard sensing and flagellar activity (72, 80). This form of locomotion, characterized by efficient self-propulsion in response to changes in the surrounding environment, renders bacteria autonomous living systems capable of finding their optimal growth conditions (81, 82), potentially within the TME.

*Taxis guided self-propulsion:* Targeted swimming towards environments that favor bacterial growth, known as taxis, requires highly organized coordination between the sensory system and flagellar motor (81, 83). This is achieved through a two-component regulatory system (TCRS) composed of sensors and response regulators (84, 85). Signal transduction linking these two components only leads to a beneficial behavioral response when it is able to sense spatial variations of relevant compounds. Due to the small size of a single bacterium (on the order of 1 μm), random swimming over large distances is necessary for sampling the environment and detecting gradients (83, 85). This continuous explorative cycle of sense and move is manifested in different forms such as the run-and-tumble motion in *E. coli* (86), or the forward, reverse, and flick pattern in *Vibrio alginolyticus* (87, 88).

The taxis behavior of bacteria can be grouped in two general categories: classical chemotaxis where the response only depends on the presence of chemical stimuli, and energy-taxis, such as aerotaxis and phototaxis, in which the cellular metabolism of the stimuli and the energy level regulate the swimming behavior (89). While membrane-spanning chemoreceptors, known as methyl-accepting chemotaxis proteins (MCPs), bind to specific attractant or repellant chemoeffectors, energy transducer proteins interact with the electron transport system to sense the energy level. The transduction pathways of bacterial taxes converge further down the signaling network where, for example, in *E. coli* the phosphorylated CheY protein interacts with
the flagellar motor, which switches the direction of rotation of the motor (85, 89). Although a general understanding of the signal transduction pathway has been elucidated from studying *E. coli* and *Salmonella*, sensory systems with higher complexity featuring more diverse receptors have been identified in other genera (81, 83).

Due to the viscous-dominated low Reynolds flow regime experienced by these microorganisms, they have evolved to rely on molecularly-driven rotation of helical flagella for propulsion (90, 91). These slender organelles are efficient at converting rotational motion into translational displacement by generating drag-induced thrust (92, 93). Molecular motors are powered by electrochemical gradients of protons or sodium ions to rotate the flagella at frequencies of up to 300 Hz (for H+) and 1300 Hz (for Na+) (94), leading to swimming velocities in the range of tens and hundreds of μm/s, respectively. Depending on the species and configuration of flagella, different strategies have been observed for changing direction in fluids and more complex media such as mucus and biofilms (82, 83).

*Tumor targeting mechanisms:* Taxis-guided self-propulsion has motivated the use of these microorganisms in the treatment of hard-to-access tissues, such as solid tumors (Figure 1-5). Despite observations of tumor regression in patients with bacterial infection in early 19th century, as well as later experiments with live bacteria for cancer treatment, tumor homing of bacteria was first studied in 1955 by Malmgren and Flanigan (95). Their study showed exclusive localization of *Clostridium* in tumors following intravenous injection. Tumor colonization of this anaerobe was also observed in rat models where normal tissues only contained bacterial spores without any germination (96). Similarly, *Bifidobacterium* another anaerobic bacteria demonstrated selective tumor localization following systemic administration into mouse tumor models (97).

Most solid tumors are characterized by the presence of a necrotic core and hypoxic regions in areas distant from the closest immature blood vessel (25, 32). Therefore, low oxygen levels are considered the main driver for aerotactic migration, followed by sustained germination and colonization of obligate anaerobes, which exhibit low oxygen tolerance (98). Interestingly, when the obligate anaerobe *C. novyi-NT* was injected into mice bearing poorly vascularized non-neoplastic tissues characterized by hypoxia, no bacterial colonization occurred. This was consistent with a later finding in a rat ischemic brain model (99). These
findings highlight the role of unique biochemical parameters of the TME in the infiltration of the solid tumor by strict anaerobes (100). Other mechanisms have been proposed for the tumor homing of facultative anaerobes that can also survive and grow under the normal oxygen concentrations present in healthy tissues (101). Studying gene knockouts indicated the role of aspartate receptors in the chemotaxis-driven tumor targeting of S. typhimurium (102). Serine receptors play a role at the start of infiltration, and directed penetration towards a necrotic core is achieved through ribose/galactose receptors. In addition, signal transduction mutants were shown to lack migration towards tumor tissues (103). Moreover, the correlation between both homing and intratumoral growth of bacteria with the size of the tumor tissue illustrated the key role of hypoxic viable cells in chemotaxis (104). Interestingly, Listeria employs an alternative strategy by targeting the immunoprivileged tumor tissue through infection of myeloid-derived suppressor cells (MDSCs) that infiltrate the tumor (105).

While the above-mentioned anaerobes display a range of responses to oxygen levels, other unique features of the TME facilitate the homing of all classes of bacteria in a similar manner. The role of the immune system in the homing of both obligate and facultative anaerobic strains is complex (101). All tumor targeting bacteria differentially take advantage of the immunosuppressive nature of the TME, particularly in the necrotic core (106). It also contributes to the dissemination pattern of facultative anaerobes in tumors, which is analogous to obligate anaerobes in spite of differences in physiology (107). Targeting and replication in tumors by aerobic strains further underpins the argument in favor of the critical role of defective immunosurveillance compared to the influence of hypoxia (106, 108). While accumulation of bacteria at the location of quiescent viable cells inside tumors would lead to the highest treatment efficacy (102), immune cells limit the presence tumor-infiltrating bacteria to necrotic regions. However, in vivo, the well perfused rim of the tumor, which is not colonized by bacteria, was shown to be eradicated through immune-mediated responses in immunocompetent mice (109). Overall, hypoxia development, release of bacterial nutrients, and the immune-suppressive environment in solid tumors are all intertwined with irregular leaky tumor vasculature (110). Consequently, passive entrapment of bacteria in tumor blood vessels and infiltration through endothelial gaps, which could be accelerated by TNF-α (111), has been considered as another contributing factor in the tumor tropism of bacteria (112, 113).
Targeted colonization of the tumor is the basis of almost all bacterial cancer therapies, but this process is contingent upon the homing and achievement of adequate accumulation. Accumulation is reliant on administration of a minimum amount of bacteria and the presence of a necrotic core, which is correlated with the developmental stage of the tumor (114). Both Gram-negative and Gram-positive bacteria, regardless of their oxygen requirements and pathogenicity, were able to colonize syngeneic and xenograft tumors in immunocompromised and immunocompetent mice (106). A comprehensive understanding of the necessary conditions for colonization has been the subject of various studies (106, 108, 114).

**Figure 1-5: Bacteria tumor tropism.** Multiple factors contribute to the tumor colonization by the bacteria. Abundance of bacterial nutrient, low oxygen level, and lack of immune surveillance play key roles in this tumor targeting behavior. Adapted from (1).

1.2.2 External energies for enhanced control

As originally demonstrated for non-living NP-based drug formulations, external stimuli can also serve to improve selective targeting of bacteria and other cell-based therapeutics (Figure 1-6) (11, 17). Responsiveness to internal stimuli, mainly in the form of chemotaxis and aerotaxis, enables cell-based systems to act autonomously, but at the cost of a relatively slow response and slightly random behavior (71, 115). Conversely, interactions with external cues enables faster control, which is accompanied by design complexities and potentially the need for closed-loop control. Additional modification of the cells is also necessary when responsiveness is not intrinsic to the cell type. External control confers dual-targeting
functionality to these agents as they are already equipped with intrinsic homing mechanisms. This added specificity leads to higher safety levels since the same efficacy can be achieved at lower dosages.

Among all physical stimuli, the use of magnetic energy has gained the most interest (Figure 1-6A). The human body is fully penetrable by magnetic fields which allows magnetically responsive agents to target deep-seated organs. Magnetotactic bacteria (MTB), a group of bacteria that biomineralize membrane-bound iron-rich nanocrystals called magnetosomes have strongly contributed to this interest (116, 117). Chains of magnetosomes serve as compass needles for MTB, helping them navigate along the Earth’s magnetic field while swimming towards the oxic–anoxic interface (OAI), a behavior known as magneto-aerotaxis (118–120). The idea of using MTB as trackable medical microrobots was proposed over a decade ago (121–123), and since then different strains of MTB such as Magnetococcus marinus MC-1 (124), Magnetospirillum magneticum AMB-1 (125), and Magnetosspirillum gryphiswalense MSR-1 (126) have been studied in vivo for drug delivery into tumors. In terms of navigation strategies, most studies focused on force-based field gradients (125, 126) or directing magnetic fields (123, 124, 127). Following peritumoral injection in mice bearing flank tumors, ~55% of the MC-1 exposed to directing magnetic field accumulated in hypoxic region of the tumor (124), and almost two-fold higher tumor infiltration was shown for magnetically assisted AMB-1 (125). Furthermore, the ability of bacteria to act as MRI negative contrast agents and the correlation with concentration has been demonstrated (125). Recently, using rotating magnetic field as a means of actuation of MTB to convectively enhance the transport of co-delivered nanoparticles was proposed (128, 129). Although more sophisticated control schemes, such as point-to-point closed-loop control (130, 131) or the use of a virtual magnetic monopole (132, 133) have been proposed for MTB guidance, none have been implemented in vivo to date. Given the innate responsiveness of MTB to magnetic fields, this thesis focuses on these bacteria as a model organism for magnetically enhanced transport.

Magnetically-enhanced tumor targeting can also be applied to non-magnetic living cells (Figure 1-6A) (134). This strategy requires the integration of magnetic nano/microparticles into the living system. Magnetic particles conjugated onto the surface of bacteria like E. coli (76, 135) and Serratia marcescens (136) conferred the ability to align with magnetic fields, with swimming velocities lower than that of sole bacteria. Microswimmers created by attaching
several *E. coli* to multilayer microparticles loaded with doxorubicin and magnetite were demonstrated to have mean swimming speeds of up to 22.5 μm/s and to deliver doxorubicin in 4T1 breast cancer cells *in vitro* under a chemoattractant gradient and a magnetic field (76). Similar microswimmers composed of multiple *Serratia marcescens* bacteria attached to a superparamagnetic bead were shown to have mean swim speeds of up to 7.3 μm/s and were able to be steered along 2D trajectories using low magnetic field amplitudes (≤10 mT) (136). Through genetically engineered expression of iron-storage ferritin and magnetosomes, *E. coli* and MSCs, respectively, have been rendered responsive to magnetic fields (137, 138).

Static fields alone are unable to focus magnetic agents at an interior point to create stable traps, as demonstrated by Earnshaw’s theorem (139). This, together with the rapid decay of magnetic forces resulting from field gradients, limit the application of static fields to shallow targets. While promising in small animal models, very high fields are required to achieve the same level of forces at larger scales. This scalability issue can partly be circumvented using relatively weak directional static fields (DMF) to align the motile agent in the specified direction. However, success of this guiding strategy is bounded by self-propulsion forces which may not be sufficient to overcome robust physiological barriers. Rotating magnetic fields (RMF), that also bypass the high field requirement, can serve as an attractive strategy in the absence of self-propulsion or when propulsive forces are not sufficiently strong, which is in part investigated in this thesis.

Another natural taxis with respect to external stimuli is phototaxis, which is active migration in response to light intensity (Figure 1-6A) (140). Photosynthetic bacteria (PSB) *Rhodobacter johrii* showed higher tumor accumulation in NIR irradiated tumors following peritumoral injection *in vivo* (141). Coculture with multicellular MCF-7 spheroids showed enhanced infiltration of the tumor core when subjected to NIR irradiation. Following peritumoral injection of PSB into tumor bearing mice, PSB accumulation in hypoxic regions of the tumor was increased by exposure to NIR light. Although spatial control was achieved by light-induced migration, the application of this approach is restricted to superficial tumors given the limited penetration depth of light in the body (72).
Figure 1-6: Externally enhanced tumor homing based on different types of physical cues. Direct taxis-based migration towards the tumor. Magnetotactic bacteria and engineered cells containing magnetic nanoparticles are guided using external magnetic fields (top). Photosynthetic bacteria are capable of active migration towards the irradiated sites (bottom). Adapted from (1).

1.3 Anticancer characteristics of bacteria

The innate tumor homing of bacteria, although a critical prerequisite, is not the sole motivation to use these microorganisms in cancer treatment. Different forms of antitumor activities provided by bacteria have fueled studies on these living therapeutic agents (101). Their antitumor effects stem from either innate toxicity, delivery of a cargo, or on-site production and release of drugs by genetically engineered bacteria (100, 142, 143). In addition to these cytotoxic mechanisms, bacteria trigger immune-mediated antitumor responses (100, 110). Relative contributions of these two pathways in final cancer cell death depends on the species, specific TME, and time of intervention (101, 143).
1.3.1 Intrinsic antitumor effects

Native cytotoxicity of different species has been studied and various mechanisms were proposed. *Salmonella spp.* as intracellular pathogens were shown to induce cell death through apoptosis \((144)\) and autophagy \((145)\) by production of toxins or uncontrolled replication inside the cell. They also compete for nutrients within the TME. *Listeria spp.* give rise to elevated intracellular reactive oxygen species (ROS) \((146)\) which thereby kills cancer cells. *Clostridium spp.* secrete many exotoxins such as phospholipases and hemolysins which disrupt the cell membrane and interfere with cellular functions \((147, 148)\).

These direct anticancer effects are complemented by immune-mediated cell death. Tumors colonized with *Salmonella* demonstrated secretion of interleukin-1β (IL-1β) from macrophages and DCs induced by LPS \((149)\) or phagocytosis of infected cells leading to pyroptosis \((110, 150)\). Enhanced cross-presentation of antigens to DCs through gap junctions \((151)\) and flagellin-mediated activation of CD8+ T cells and NK cells resulting in production of IFN-γ \((152)\) have also been reported for this genus \((110)\). Phenotypic alteration in MDSCs that carry *Listeria* boosts IL-12 levels, which is accompanied by a stronger antitumor response from NK and CD8+ T cells \((105, 112, 153)\). In the case of *Clostridium*, secretion of IL-6, granulocyte-colony stimulating factor, and macrophage inflammatory protein 2 is considered responsible for recruiting immune cells \((154)\) and release of TRAIL from neutrophils \((155)\).

Modification of the cell membrane surface with targeting moieties can further boost therapeutic effects by enhancing tumor accumulation. In vivo studies using an engineered attenuated RGD-displaying *Salmonella* revealed prolonged survival of mice, with 80% enduring up to 160 days after tumor inoculation. Similarly, attenuated *Salmonella* with CEA-specific antibody resulted in inhibited growth of tumors as well as local accumulation of CD3+ T cells and CD11b+ macrophages \((156)\).

1.3.2 Delivery of therapeutic payloads

Analogous engineering routes are also used to attach drug payloads onto bacterial membranes \((157, 158)\). Nguyen et al. developed a cargo-carrying system featuring tumor targeting bacteria *S. Typhimurium* and paclitaxel (PTX)-loaded micro-liposomes \((159)\). The average velocity of this kind of “bacteriobot” was reduced to 3 µm/s. In their final study, these bacteriobots selectively reduced the viability of cancer cells *in vitro*. Another example was
developed by Park et al. who attached polystyrene carboxylated microbeads onto attenuated S. typhimurium (160). Chemotaxis-driven migration speeds of ~30 μm/min were observed for the bacteriobots when exposed to lysates or cancer cell spheroids while their velocity reached only ~5 μm/min in the presence of normal cells. In another study, the radioisotope 188Rhenium was conjugated to attenuated live Listeria monocytogenes via strain-specific antibodies (161). Mice with metastatic pancreatic tumors were injected intraperitoneally and bacterial proliferation was found in metastatic sites, while healthy tissues was spared. Daily low dose injections for sustained delivery of radioactivity resulted in a 90% reduction of the number of metastases compared to 50% for Listeria alone. The measured radioactivity level in the liver and kidneys was not detectable by day 7. Suh et al. developed a biohybrid autonomous drug delivery platform composed of PLGA nanoparticles (NPs) conjugated to an attenuated auxotrophic mutant of S. Typhimurium (Figure 1-7A) (162). This chemotaxis-defective strain demonstrated significantly better tumor penetration in vitro compared to NPs alone, and their conjugation resulted only in a slight decrease of this ability. Intercellular transmigration was determined as the dominant mode of translocation inside tumor masses highlighting the role of TME in tumor penetration of bacteria. Invasion assays also revealed a decreased number of bacteria invading the cells when coated with polyethylene glycol (PEG) or carrying conjugated NPs.

Similarly, therapeutic cargos have been integrated with bacteria that are innately responsive to external physical cues for enhanced delivery at the tissue scale. A similar strategy has been adopted for non-responsive bacteria where they are modified with nanomaterials to allow for external control. Taherkhani et al. investigated the potential of the MTB strain MC-1, as a drug carrier (163). MTB-liposome conjugates were synthesized by utilizing the amine groups on the surface of the Gram-negative bacteria. Reduced motility was correlated with the number of attached liposomes and residence time in buffer. The same system was employed in vivo to target the hypoxic core of tumors under directing magnetic fields (124). Another strain of MTB, AMB-1, was also employed as a magnetically controllable motile carrier. Here, integrated indocyanine green nanoparticles (INPs) rendered this biohybrid platform capable of imaging and photothermal therapy (Figure 1-7B) (125). Average mean velocity of this biohybrid microrobot was reported to be 13.3 μm/s. They were peritumorally injected and guided using magnetic field gradients. Photothermal therapy using NIR irradiation inhibited tumor growth, and no significant changes in biochemical parameters associated with liver and
kidney function implied acceptable safety levels. In addition to chemically conjugated payloads, DOX-internalized MTB was also recently presented, which was motivated by chelation of DOX with Fe$^{3+}$ (126). Drug-internalized MSR-1 maintained their motility for 3 hr. In vivo, this motile platform was characterized with better targeting and higher tumor growth suppression compared to inactivated bacteria and free DOX. The same strain has been also tested for applications in magnetic hyperthermia (164). Similarly, external control has been presented for bacteria that are either engineered to synthesize or coupled to magnetic particles (76, 136, 137).

While most studies have focused on the application of magnetic fields, the responsiveness of bacteria has been also exploited for other types of stimuli. Zheng et al. proposed living PSB as an ideal carrier possessing both hypoxia targeting and NIR phototaxis which could be leveraged for photothermal therapy (Figure 1-7C) (141). While PSB alone suppressed tumor growth after 10 days, which was attributed to nutrient competition, irradiated PSB resulted in much smaller tumor sizes.

1.3.3 Genetically engineered bacteria for higher efficacy

A third group of bacteria-based therapeutics comprises genetically engineered bacteria with encoded functionalities to enhance targeting and therapeutic efficiency. These genetic modifications were implemented both in probiotic and pathogenic strains. In a pioneering work by Pawelek et al., different auxotrophs of engineered Salmonella were explored as anticancer vectors (165). Engineered auxotrophs expressed the HSV TK gene which led to activation of the prodrug ganciclovir and suppressed tumor growth. Similarly, attenuated S. Typhimurium was genetically engineered to produce IL-2 by cloning the human gene for IL-2 into a plasmid (166). Mice bearing metastases in the liver demonstrated significantly lower number of metastases when gavaged with IL-2 expressing bacteria in contrast to an attenuated strain without IL-2. These bacteriobots were able to colonize the tumor for at least 4 weeks. More recently, Leventhal et al. developed a therapeutic platform based on non-pathogenic E coli Nissle (EcN) that activated the STING pathway (167). Reduced tumor growth was observed in melanoma-bearing mice treated with intratumoral injections of EcN. Triggering localized inflammation by intratumoral production of the STING-agonist cyclic diAMP resulted in better tumor regression compared to control EcN.
Further genetic circuits have been introduced to control production and release of the therapeutic agents. Camacho et al. engineered *Salmonella* with an inducible autolysis system leading to lysis of bacteria triggered by anhydrotetracycline (168). This system was complemented with aspirin-dependent production of the therapeutic agent, and sifA mutation which allowed the bacteria to escape the vacuoles when internalized by the cells. Infection of cancer cells with engineered bacteria producing Cp53 confirmed proliferation of this strain inside cells and Cp53-induced cell death. In a microfluidic model of perfused tumor tissue, *E. coli* expressing Trz1, a glucose-sensing receptor (169), were shown to detect glucose gradients and express green fluorescent protein (GFP) in response (170). Based on this data, mathematical models predicted treatment of 99.2% of cells within a tumor using Trz1-activated drug delivery compared to 70.8% with similar systemically delivered drugs.

Incorporating inducible elements sensitive to oxygen levels is another attractive strategy due to its potential in localizing the antitumor effects. In a study by Anderson et al., environmental cues, including cell density, hypoxia, and inducible inputs, were used to control the internalization of *E. coli* (171). This response was achieved by placing the expression of the invasin gene from *Yersinia pseudotuberculosis* under the regulation of the quorum sensing lux operon, a hypoxia-induced fdhF promoter, or an arabinose-inducible araBAD promoter. This resulted in 8% of added bacteria being recovered following lysis of cancer cells expressing β1-integrins, while levels of bacteria without invasin expression were undetectable. Similarly, *S. typhimurium* with a gene encoding the production of a cytotoxic protein (HlyE) under the control of a hypoxia-inducible promoter (FF+20*) colonized hypoxic tumor regions after systemic administration (172), leading to a reduction in tumor growth and an increase in tumor necrosis. *S. Typhimurium* was also programmed in another study to solely survive in anaerobic conditions by placing an essential gene, asd, under the regulation of a hypoxia-conditioned promoter (173). *In vivo*, the intravenously administered engineered strain successfully colonized tumors and repressed growth, while undergoing lysis and clearance in normal tissues. Mengesha et al. developed a hypoxia-inducible promoter (HIP-1) derived from the *Salmonella* pepT promoter (174). Following systemic administration of the bacteria, the reporter gene expression for GFP and RFP was restricted to the hypoxic tumors and was approximately 15-fold higher compared to a constitutive promoter.
Another intriguing opportunity is engineering the therapeutic response as a function of quorum sensing – the ability of some bacteria to regulate gene expression in response to density of the cell population. The bacterial quorum sensing autoinducer-2 (AI-2) signaling pathway of *E. coli* was rewired to enable programmed motility, sensing and payload delivery based on the density of epidermal growth factor receptor (EGFR) (175). *In vitro*, the engineered bacteria had the ability to survey surfaces to distinguish between diseased and healthy cells, and initiate DsRed gene expression in response. *E. coli* Nissle 1917 was engineered to produce and release nanobodies targeting programmed cell death–ligand 1 (PD-L1) and cytotoxic T lymphocyte–associated protein-4 (CTLA-4) intratumorally once a quorum was reached (176). Compared to clinically relevant antibodies, a single dose of intratumorally injected engineered bacteria resulted in an increase in activated T cells, tumor regression, as well as an abscopal effect. Using similar intratumoral quorum lysis, a non-pathogenic *E. coli* strain was engineered to lyse and release a nanobody antagonist of CD47, a frequently overexpressed anti-phagocytic receptor, within the tumor (Figure 1-7D) (177). Delivery of the nanobody by intratumoral and intravenous administered bacteria increased tumor-infiltrating T cell activation and induced tumor regression. Harimoto *et al.* presented a bacteria-in-spheroid coculture (BSCC) platform to screen *S. typhimurium* strains engineered with a variety of synthetic gene circuits for the expression and triggered release of a range of antitumor therapeutics (178). Candidates displaying significant therapeutic efficacy in the BSCC platform, such as azurin, theta-toxin, and hemolysin E, were shown to have similar efficacy *in vivo*. 
Safety concerns and clinical translation in bacterial cancer therapy

Despite unique benefits offered by tumor targeting bacteria, use of living bacteria for cancer therapy has encountered hurdles mainly associated with their potential toxicity as pathogens. Bacterial infections are known as the most frequent cause of sepsis, an unusually severe response from the immune system in form of a systemic inflammation. This
complication, if not treated, can lead to fatal oxygen deprivation and organ failures. However, thanks to better understanding of the TME and advances in areas like synthetic biology and genetic engineering, researchers have been able to address some of the concerns regarding the safety aspects. In addition, other factors such as the route of administration and dosage heavily influence the immune response which can be tuned on the basis of pathogenicity of the species used for the treatment.

In general, strategies to improve the safety of bacterial therapeutics can be categorized into two types of approaches. One set of techniques focuses on reducing the pathogenicity of these microorganisms while another strategy is aimed at improving the targeting efficiency. The former results in formation of attenuated strains, and the latter allows for lower dosage while achieving the same antitumor effect. There has been a paradigm shift over the past few years where use of probiotic bacteria has been investigated for cancer treatments. This group of bacteria is considered safe to orally consume and offers benefits to the host in context of gastrointestinal diseases. Their potential in other indications and positive effects in combination with other forms of immunotherapy is an emerging area of research.

Bacteria can be engineered in different ways to improve their safety profile as anticancer agents. Genetic modifications regarding diminishing the virulence of tumor targeting bacteria offers opportunities to provide safer strains. *C. Novy*-NT represents an attenuated strain of pathogenic *Clostridium novy* without its secreted lethal toxin which proved to be an effective strategy in reducing the toxicity (179, 180). This strain has been examined in several clinical trials to develop bacteria-based cancer therapeutics. As lipopolysaccharides (LPS), major constituents in the outer membrane of gram-negative bacteria, is known for triggering potent immune response, modification of the membrane or camouflaging have been also proposed to control the pathogenicity of the bacteria. For instance, *S. typhimurium* VNP20009 feature altered membrane composition due to deletion of the *msbB* gene leading to lower risk of TNF-α mediated septic shock (181). In addition, stable *purl* gene removal in this strain aims to reduce the virulence (182). Safety of this strain is also confirmed through clinical trials (183, 184).

It should be noted that antitumor activity of bacteria is often compromised through attenuation of their virulence. Finding a balance between the safety and efficacy is therefore a key consideration in all these modifications. Even bacteria with low native cytotoxicity can still serve as an attractive vaccine vector. More recently, to avoid permanent attenuation of bacteria,
*E.coli* Nissle 1917 was programmed to express dynamic encapsulation to temporarily evade the immune system leading to 10 fold increase in the tolerated dose by mouse models (185).

Strategies to reduce systemic toxicity by enhancing the targeting efficiency also takes different forms. One strategy is the use of auxotrophic mutants in which modified strains only proliferate in the presence of an additional nutrient. Enriched compounds exclusive to TME have been selected for this purpose resulting in relatively weaker proliferation in off-target areas. For example, conferring leucine and arginine auxotrophy to *S. typhimurium* resulted in higher survival rate in non-tumor bearing mice and tumor regression in the treated mouse models (186). Surface display of targeting moieties with the goal of specific tumor binding falls into the same category. Arg-Gly-Asp (RGD) peptide on the outer membrane of an attenuated *S. typhimurium* gave rise to substantial increase of accumulation in xenografts overexpressing \( \alpha v3 \) (187). Targeting tumor associated antigen, carcinoembryonic antigen (CEA) (156) and CD20 (188), through anchoring corresponding antibodies on the surface of differently modified *S. typhimurium* strains has been shown to improve the localization of bacteria. Synthetic adhesins have been also utilized as tumor-specific binding proteins on the surface of *E.coli* where lower required dose for tumor colonization was reported (189).

In addition to efforts to confine the presence and proliferation of bacteria, similar enhanced targeting is achieved through spatiotemporally controlled release of the payload. Different means for triggering the promoters have been proposed such as tumor-specific properties like hypoxia, quorum sensing, exogenous chemical transcriptional factors, and radiation. Higher degree of control is achieved when multiple inducers of different types are combined. Incorporating a QS circuit in *E.coli* with either hypoxia or arabinose-induced promoter resulted in expression of invasin only at high cell densities and in the presence of the inducer (171). Linking synchronized lysis circuit (SLC) to the QS system in *E.coli* were also carried out more recently (177, 190).

Lastly, any externally enhanced transport to the tumor discussed in the previous section potentially contributes towards improving the safety of the cell-based platforms. By reducing the required dose to achieve the same therapeutic outcome, these strategies similarly lower the risk of systemic toxicity. Therefore, magnetically responsive bacteria can not only offer controllable vectors for delivery, but also potentially lower barriers associated with translation of bacterial therapeutics into the clinic.
Supported by these advances, many clinical studies have been performed in the field of bacteria cancer therapy and some are currently in progress. Inconsistent colonization due to suboptimal choices of preclinical models and cohort of patients along with the host toxicity have been reported to be the main causes of terminated clinical trials. In addition, tumor relapse following incomplete eradication of the cancer cells further impeded the clinical development. Using bacteria-based therapeutics as part of a combination therapy and developing more sophisticated genetic modifications for precise control over the growth and release of the drug from bacteria have been current stage of research to address those challenges.

1.5 Structure and aim of the thesis

The body of work represented in this thesis is united by its aim of studying MTB as a model organism for magnetically enhanced, cell-based drug delivery. Particular focus is placed on enhancement of transport and associated mechanisms in the context of cancer therapy. Nevertheless, therapeutic targets could be potentially extended to other ailments in which targeting a hard-to-access area is of central importance such as intravascular obstructions or central nervous system disorders. Since microfluidic transport enhancement is also of interest in non-medical applications where physical access is restricted, microfluidic pumping using MTB as an effective ferrofluid is also introduced.

A central theme that helps inform the structure of this document is the relationship between scale, physical phenomena (fluidic and magnetic), and instrumentation. In its chapters, it proceeds along experimental steps that reflect different aspects of the in vivo journey of the MTB toward drug delivery in tumors. Although some phenomena such as gravitational settling in a dense suspension are of negligible importance in vivo, the symbolic scheme shown in Figure 1-8 summarizes different types of behaviors that these microorganisms exhibit under various conditions. The steps taken in the thesis start with a concentrated uniformly distributed population of bacteria that generate flow through collective convective effects. It continues with occasions in which a non-uniform distribution would emerge over time. The suspension of MTB continuously dilutes as the bacteria are rapidly distributed through the body along different routes.

Analogously, various transport mechanisms are associated with each stage, and separate chapters have been dedicated to study these behaviors. Chapter 2 discusses the physical
principles governing a dense uniform population of bacteria suspended in a liquid. It provides both computational and experimental methods to assess the characteristics of MTB suspension as living ferrofluids, accompanied by a comparison to a synthetic counterpart. This behavior is observable at time scales less than approximately 30 min, in which density gradients are not present in the suspension.

Chapter 3 examines various in silico, in vitro, and in vivo assays employed to investigate transport mechanisms in physiological models under rotating magnetic fields. The transition from flow mediators to individual surface walkers was first studied computationally. Modeled as individual entities, bacteria undergo torque-driven surface exploration enabling translocation across endothelial barriers. Unlike collectively driven convective flow, this mechanism is applicable when dealing with suspensions at lower concentrations after longer time periods. Traversing tissue barriers was further studied in microfluidic platforms in which suspensions of MTB were magnetically actuated and encountered tissue interfaces comprised of endothelial linings and collagen matrices. The chapter concludes with the results of an in vivo trial in which magnetic actuation was locally applied to tumors in a mouse model.

Chapter 4 introduces a novel control scheme for selective actuation of the bacteria at target areas with favorable prospects for clinical scalability. The fact that drug carriers rapidly disperse inside the body following systemic administration necessitates actuation strategies that inherently localize the desired effects at remote points. The chapter first discusses the physical principles that can satisfy this need in the context of magnetic manipulation, followed by in vitro assays exhibiting successful execution of this targeted manipulation strategy. A major part of this chapter is dedicated to the design and fabrication of an animal scale selection field setup intended for future in vivo studies.

Chapter 5 concludes the thesis by summarizing the findings drawn from studying MTB as model system in the context of cancer therapy and highlighting the key theoretical and experimental methods that were developed. Finally, it offers perspectives on outlook, emphasizing discussion of next steps toward optimizing this delivery system and extending these findings to adjacent bacteria-based platforms.
Figure 1-8: Thesis overview. Depending on the spatial scale of actuation, time scale of the treatment, and concentration of the bacteria, different regimes of actuation can be envisioned for varying roles of bacteria as microrobots. In vivo journey of the bacteria is symbolically adopted as the thread of this thesis to dissect different transport mechanisms associated with magnetically responsive organisms.
2 Ferrohydrodynamic phenomenon in living ferrofluids

This chapter is adapted from (128). The author has performed all the experiments and simulations in this study. The author also acknowledges the contributions from Michael Christiansen in the design of the proposed magnetic setups and actuation schemes in this study.

Magnetic actuation offers a means to wirelessly control flow in ferrofluids for applications including microfluidic pumping and targeted drug delivery. Despite the promise of these concepts, practical use of synthetic ferrofluids as actuators of flow frequently requires high concentrations and is hindered by low ferrohydrodynamic coupling efficiency and inhomogeneous flow fields. Inspired by the magnetic properties and hydrodynamic forms displayed by magnetotactic bacteria (MTB), this work studies the use of these microbes as a living, self-replicating ferrofluid for improved fluidic transport via magnetically coerced rotation. Using multicore iron oxide nanoparticles as a performance benchmark, MTB under rotating magnetic fields are shown to produce more homogeneous and efficient flow. Coupling is enhanced whether the comparison is made in terms of volume of magnetic material or total volume fraction. To clarify the mechanistic role of interactions with boundaries in transport, a computational model is developed and validated experimentally. Applying this model, two distinct and feasible magnetic control strategies are predicted: a rotating gradient field that generates directional flow despite boundaries that promote flow in opposing directions, and a magnetostatic gating field that enables spatially selective actuation. The advantageous properties identified for MTB open a design space for these strategies to be realized.

2.1 Introduction

Ferrofluids, colloidal suspensions of magnetic particles, are materials that rely on a combination of magnetic and fluidic characteristics to perform their various functions (191, 192). The possibilities they offer for magnetic actuation make them uniquely valuable in circumstances where direct contact is undesirable or impractical, such as controlling microscale
systems or accessing deep physiological targets (193–196). Ferrohydrodynamic coupling, a phenomenon that depends just as much on hydrodynamic as magnetic properties, allows these materials to translate magnetic stimuli into flow (197–199). This flow can be harnessed either to transport the surrounding liquid or the magnetic component of the ferrofluid (191). In this context, interaction between ferrofluids and magnetic stimuli has been suggested as a contactless mechanism for microfluidic pumping or delivery of therapeutic agents to sites of disease (200–204).

Although attention has been drawn to the potential of synthetic ferrofluids as actuators of flow (205–209), in practice these approaches typically require high concentrations. This runs contrary to ideal performance, in which the volume fraction of the solution conveyed during pumping is maximized by reducing the volume fraction of flow mediators. Moreover, functional materials and transport strategies compatible with low concentrations are particularly desirable in instances where high concentrations are unattainable, such in the context of in vivo drug delivery. Interparticle dipole-dipole interactions occurring in high concentrations of synthetic ferrofluids can also result in the formation of irreversible aggregates that produce spatially and temporally inhomogeneous flow. Overcoming these limitations requires reexamining hydrodynamic properties, and nature offers forms well adapted to fluidic coupling at various length scales and environments. Bacteria, for example, have served both as inspiration for wirelessly controlled synthetic, microrobotic devices (92, 129, 210–212) and living materials enabling transport (72, 124, 213, 214). Magnetic manipulation provides a means to leverage these favorable hydrodynamic forms while exceeding the forces and torques offered by the natural propulsive mechanisms of microbes, a factor limiting their potential for active transport.

Here, we describe the use of magnetotactic bacteria (MTB) as a living, self-replicating ferrofluid for fluidic transport via magnetically coerced rotation. The approach marries the favorable hydrodynamic characteristics of bacteria with a scalable magnetic stimulus to wirelessly transfer torques higher than those involved in natural swimming behavior. As a basis of comparison, we employ iron oxide nanoparticles (IONPs) that self-assemble into high aspect ratio microstructures upon exposure to the same magnetic stimulus (Figure 2-1). The increased hydrodynamic radius of the MTB is shown to lead to enhanced coupling efficiency when normalized to the quantity of magnetic material, reduced aggregation after actuation, and more
homogeneous flow. We develop and validate a computational model to more fully understand the behavior of MTB as a living ferrofluid, and to clarify the role of their interaction with surrounding boundaries. Using this model, we predict the possibility of two distinct magnetic stimulus strategies: as a localized microfluidic pump and as means of drug delivery with spatially selective actuation. The unique and advantageous properties identified for MTB open a design space for such strategies to be realized.

2.2 Magnetic characterization of living and synthetic flow mediators

Growth and responsiveness of the MTB needs to be monitored and tracked as they are cultured to find the optimum timepoint for using them as a flow mediator. Optical density (OD) of the solution at 600 nm was measured under no external field, from which the MTB concentration can be obtained using an established calibration curve. Figure 2-2A shows the result of MTB proliferation in culture for 10 days. Afterward, OD values were measured when the solution was exposed to a magnetic field aligning magnetically responsive MTB in two different directions, i.e. parallel and perpendicular to light path, using a small bar magnet on top of the cuvette (Figure 2-2B). While the OD without an applied field showed the population...
growth behavior of MTB, $C_{\text{mag}}$ was calculated based on the ratio of OD under the two different field orientations to measure the bulk magnetic responsiveness of the sample (Figure 2-2A) (215), and indicated of the ratio of bacteria with magnetosomes to the ones without magnetosomes.

Considering these data, one can define an optimum timeframe at which both concentration and magnetic responsiveness for normal culture are at desirable values. It is worth noting that alternative techniques such as concentrating the sample and frequent refreshing of the culture medium can also be done to achieve the same performance from MTB harvested at other time points.

While $C_{\text{mag}}$ gives comparative information on responsiveness of the MTB population, vibrating sample magnetometry (VSM) enables us to quantify the moment of the sample. Combined with bacterial concentration, the number of MTB with magnetosomes chains can be estimated. In addition, other parameters such as saturation magnetization and the average magnetic moment of individual IONP can be extracted from VSM data. Magnetic properties of suspensions of both MTB and IONPs were investigated with VSM.

MTB solution reached the nearly saturated state at field magnitudes of around 400 Oe (40 mT) and significant deviation from the linear range starts relatively at very low field magnitudes, i.e. $\sim 2$ mT (Figure 2-2C). Corresponding saturation behavior of IONP suspension occurred at around 2500 Oe (250 mT) (Figure 2-2D). For this synthetic ferrofluid, linear relationship in low magnetic field limit was still valid at comparatively higher field magnitudes.
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Ferrohydrodynamic pumping in living ferrofluids

Figure 2-2: Characterization of MTB solution and synthetic ferrofluid.  
A) Time-evolution of the optical density at 600nm (indicator of number concentration) and $C_{mag}$ value (indicator of magnetic responsiveness) for MTB culture after passaging.  
B) Schematics of OD and $C_{mag}$ measurement principles.  
C) $M$ vs. $H$ curve of the MTB solution obtained by VSM.  
D) $M$ vs. $H$ curve of the IONPs suspension obtained by VSM.

2.2.1 Effect of culture days and magnetic order in chains

In order to better understand the magnetic properties observed in MTB, magnetic properties of samples at different time points after passaging were also measured by VSM. This approach enabled us firstly to monitor how the quantity of magnetic material contained in magnetosomes in a population of bacteria changes over time, regardless of bacteria without magnetosomes, and secondly to verify the presence of magnetic order along the chain. In other words, synthesis of magnetosomes and degree of their interaction can be quantified indirectly.

Samples were measured 3, 6, and 10 days after passaging. Before measurement, MTB culture were spun down and the pellet was resuspended in 1 ml PBS. Considering that MTB were still at the end of the log phase after three days, the magnetosome concentration was too low for a consistent signal to be measured. However, as the VSM data indicate in Figure 2-3A, an increase in net magnetic moment of the MTB samples was seen as they remained longer in
culture. Because $C_{mag}$ measures the fraction of bacteria that are magnetically responsive, whereas VSM measures the total amount of magnetic material, the observed difference in trends between days 6 and 10 can be attributed to the elongation of preexisting magnetosome chains and proliferation of bacteria without magnetosomes as iron is depleted from the culture medium. Furthermore, considering the size of nanoparticles contained in the magnetosomes, they can be seen as single domain nanoparticles for which net magnetization is directly correlated with the order of their magnetic moments. To better understand and verify the presence of magnetic order along the chain of magnetosomes, MTB samples were also suspended in gels to become physically fixed. Again, magnetic response of samples were measured by VSM and one could easily see hysteresis in $M$ vs $H$ curve, implying magnetic order along the chains even in the absence of external field (Figure 2-3B).

This is consistent with the well-known role of magnetosome chain as magnetic compass by which MTB are able to navigate through the Earth’s magnetic field. Magnetosomes, organelles formed by biomineralization of iron oxide crystals (with the size range of 20-50 nm for AMB-1) bounded by a lipid membrane, would enable efficient magnetotaxis only when they are highly ordered in chain-like structures and yield a net magnetic moment. Such arrangement maximizes the torque attainable at each external field magnitude, which also makes them favorable as flow mediators.

Figure 2-3: $M$ vs $H$ curve for MTB solution obtained by VSM at different time points of the culture. A) Effect of culture days on magnetic response of MTB suspended in PBS where they freely rotate. B) Effect of culture days on magnetic response of MTB fixed in a gel where noticeable hysteresis was observed.
2.2.2 VSM data analysis

Considering the VSM data for MTB and the fact that iron oxide core size of magnetosomes falls in the range of single domain NPs, an attempt was made to fit well-known Langevin function \( L(\alpha) = \coth(\alpha) - 1/\alpha \) to the obtained VSM data as dependence of effective relaxation times are usually given as the function of normalized magnetic energy i.e. Langevin parameter \( \alpha = \frac{\mu_0 m H}{kT} \) (216–218). As shown in Figure 2-4A, taking \( m \) as the fitting parameter, a relatively good fit was obtained assuming \( m \approx 2.3 \times 10^{-18} \) A m² for MTB. This value corresponds the moment of single magnetite NP of 21 nm diameter, implying that VSM data reflects properties of the individual particles of the magnetosomes. A possible explanation for the contradiction posed by observation of remanence in MTB gel (implying ordering along the chain) yet also behavior dominated by isolated magnetosome particles in liquid (seemingly contradicting ordering along the chain) centers on agitation of the sample at a frequency on the order of 10s of Hz by the VSM. At this frequency, strong mechanical coupling with the solution is expected and would result in orientations of the bacteria that are more random compared to the orientations they assume under similar fields when not being vibrated (219). This coupling can affect the magnetic response of IONPs almost in a same way as fitting indicated \( m \approx 3 \times 10^{-19} \) A m² corresponding to the moment of single maghemite NP of 12 nm diameter. As a result, VSM data are corrected for this effect by scaling the x-axis (208) using the average number of magnetosomes per chain for MTB (220, 221) and theoretical results for multicore IONPs (222).

\[ \text{Figure 2-4: Fitting Langevin function to obtained VSM data for A) MTB and B) IONP suspensions. Fitted curve implies that VSM data mostly represent magnetic response of isolated nanoparticles present in both samples.} \]
The above-mentioned contradiction would be observed when magnetic moments of flow mediators were estimated using the M vs H curve in the vicinity of zero field magnitude. Using this approach, the moment is given by:

\[
m = \frac{3k_B T}{\mu_0} \left( \frac{dM}{dH} \right)_{H \to 0}
\]

Following this approach, moments of single magnetic materials inside both ferrofluid samples were calculated as follow:

\[
m_{\text{MTB}} = \frac{3 \times 1.38 \times 10^{-23} \times 298}{4\pi \times 10^{-7}} \times 5.8 \times 10^{-4} \approx 5.7 \times 10^{-18} \text{ A.m}^2
\]

\[
m_{\text{IONP}} = \frac{3 \times 1.38 \times 10^{-23} \times 298}{4\pi \times 10^{-7}} \times 5.1 \times 10^{-5} \approx 5.0 \times 10^{-19} \text{ A.m}^2
\]

These values were expectedly close to previously obtained values as this approach basically relies on fitting the slope near zero for randomly oriented anisotropy. However, saturation magnetization could be used to extract relevant information as it is less affected by the mechanical coupling induced by the vibration. In equilibrium, saturation magnetization \( M_s \) and \( H \) are collinear and represents a condition in which flow mediators are aligned with external field. For a ferrofluid,

\[
M_s = nm = M_d\phi
\]

where \( n \) is number density of flow mediators and \( \phi \) represents volume fraction of the magnetic material inside the suspension. Each nanoparticle has moment \( m = M_dV_p \) in which \( V_p \) shows the nanoparticle volume and \( M_d \) represents the magnetization. For magnetite nanoparticles inside the magnetosomes, \( M_d = 4.46 \times 10^5 \text{ A/m} \) or \( \mu_0 M_d = 0.56 \text{ T} \) and for maghemite of which the multicore IONPs are made \( M_d = 3.95 \times 10^5 \text{ A/m} \) or \( \mu_0 M_d = 0.49 \text{ T} \) were assumed. Applying these assumptions to the VSM data, one can extract the average moment per each flow mediator inside the sample. It should be noted that for MTB, moment per single bacterium was obtained using the number density of bacteria inside the solution.

\[
\text{OD} \approx 1.4 \rightarrow n \approx 1 \times 10^9 \frac{\text{cells}}{\text{ml}}
\]

\[
M_s = \frac{6.6 \times 10^{-5} \text{emu}}{100\mu\text{l}} = 6.6 \times 10^{-4} \text{emu/cm}^3 = 6.6 \times 10^{-7} \frac{\text{A.m}^2}{\text{ml}} = 1 \times 10^9 \frac{\text{cells}}{\text{ml}} \times m
\]
\[ m_{\text{MTB}} = 6 \times 10^{-16} \text{ A.m}^2 \]

Also, same calculations can be done for IONPs inside the synthetic ferrofluid.

\[
n = \frac{2.2 \times 10^{14}}{1 \text{gr particles}} \times \frac{2.5 \text{mg particles}}{\text{ml}} \rightarrow n = 5.5 \times 10^{11} \text{ particles ml}^{-1}
\]

\[ M_s = \frac{2.93 \times 10^{-2} \text{emu}}{100 \mu l} = 2.93 \times 10^{-4} \text{emu/cm}^3 = 2.93 \times 10^{-4} \text{ A.m}^2 / \text{ml} \rightarrow 5.5 \times 10^{11} \text{ particles ml}^{-1} \times \text{m}
\]

\[ m_{\text{IONP}} = 5.3 \times 10^{-16} \text{ A.m}^2 \]

Furthermore, using \( M_s = M_d \phi \), one can estimate the volume fraction of magnetic materials inside the suspension. Following this approach for both samples, we can compare the corresponding values for living and synthetic ferrofluid samples:

\[
M_s = 4.46 \times 10^5 \frac{\text{A.m}}{\text{m}} \times \phi_{\text{MTB}} \rightarrow \phi_{\text{MTB}} = \frac{0.66}{4.46 \times 10^5} \approx 1.5 \times 10^{-4}\%
\]

\[
M_s = 3.95 \times 10^5 \frac{\text{A.m}}{\text{m}} \times \phi_{\text{IONP}} \rightarrow \phi_{\text{IONP}} = \frac{293.5}{3.95 \times 10^5} \approx 7.4 \times 10^{-2}\%
\]

It is important to note that this volume fraction only represents the magnetic materials contained in the samples. To obtain volume fraction of the net material, a Multisizer was used to measure the number density of bacteria for which volume of single bacterium is measured as it passes through the aperture. Average total volume of bacteria in 500 \( \mu l \) of a 1:1000 diluted MTB suspension was measured 290,000 mm\(^3\) which is equivalent to the volume fraction of:

\[
\phi_{\text{MTB}} = \frac{290,000 \times 10^{-18} \times 1000}{500 \times 10^{-9}} \approx 5.8 \times 10^{-2}\%
\]

For the synthetic ferrofluid, this variable can be obtained using the number density of the particles and therefore the volume fraction of multicore IONPs can be estimated:

\[
\phi_{\text{IONP}} = nV_p = 5.5 \times 10^{11} \times 10^6 \times \frac{\pi (210 \times 10^{-9})^3}{6} \approx 2.5 \times 10^{-1}\%
\]

### 2.3 Hydrodynamic characterization and quantification of magnetically induced flow

To provide adequate context for the characterization of hydrodynamic properties of MTB under conditions of magnetically induced flow, we performed a systematic comparison with a synthetic ferrofluid commonly employed in previous studies of ferrohydrodynamic
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Suspending MTB were confined in customized PDMS wells and exposed to a uniform rotating magnetic field (RMF) (Figure 2-12A, B), rotating around the y-axis (Figure 2-1, Figure 2-5A). Maintaining constant field magnitude, frequencies were swept upward over the range of 0 to 60 Hz, depending on the sample and field magnitude, and the generated flow velocities were studied by particle image velocimetry (PIV) analysis of recorded actuation sequences (Figure 2-5B). It should be noted that all experiments were conducted well below the self-replication time of MTB to ensure a constant concentration.

Average magnetically induced velocity across the whole field of view was quantified for MTB solutions at different actuation frequencies under three external field magnitudes (Figure 2-5C), revealing an expected increase in velocity with increasing frequency until a peak is reached and the fluidic drag forces overcome the applied magnetic torque. The peak value for velocity increases with increasing magnetic field magnitude and applied magnetic torque. However, above approximately 10 mT, magnetization of the MTB begins to appreciably saturate and equilibrium magnetization behavior deviates significantly from linear susceptibility (Figure 2-2). This helps to explain why the overall increase in the induced velocity was more pronounced between 4 mT and 12 mT than between 12 mT and 20 mT. Below saturation, subpopulations of MTB with the longest magnetosome chains contribute disproportionately to the observed behavior, which may account for the broadening of the peak at 20 mT. To emphasize the possibility of employing low, scalable field magnitudes, 12 mT was chosen as the field magnitude for actuation of the samples in the rest of this study. Suspensions of IONPs were subjected to the same experimental conditions, revealing frequency dependence differing from the MTB solution, with a wider distribution of frequencies associated with peak induced velocity and peak frequencies that decrease with increasing field magnitude (Figure 2-5D).

The flow versus frequency curves for both ferrofluids at each amplitude consistently exhibited a peak, corresponding to conditions of maximal hydrodynamic coupling between the suspended MTB or IONPs and their surrounding fluid. Combined with other characterization data and a model for the dynamic magnetization response, this frequency can be used to infer effective hydrodynamic sizes. A description of magnetic torque as resulting from phase lag between the magnetization of volume elements of a sample and the external applied field is phenomenological and general, but mechanistic approaches that connect this behavior to
material properties require assumptions about magnetic relaxation processes. Typically, under time-varying magnetic fields, mechanisms such as Néel and Brownian relaxation are invoked as the processes determining the timescale of alignment of IONPs’ magnetization with the external field (224, 225). These mechanisms, which describe stochastic reversal in the absence of an applied field through internal magnetization reorientation or physical rotation, respectively, are only strictly valid within the limit of low field magnitudes and noninteracting particles in the form in which they are typically applied. Although ferrohydrodynamic coupling is rarely studied experimentally under such conditions, the useful underlying message of these mechanistic models is that the phase lag and maximal coupling occur when the actuation frequency, \( \omega \), corresponds to the timescale of relaxation, \( \tau \) (207, 223), i.e.:

\[
\tau \Omega \approx 1
\]  

As will be further discussed in section 2.5, physical rotation is expected to dominate the magnetization response of relatively large IONPs and strongly magnetically interacting aggregates suspended in a liquid (208, 225), making Brownian relaxation most relevant to the ferrofluids studied here. For low applied fields, the value of its time constant is approximated by (208, 226, 227):

\[
\tau_B = \frac{3\eta V_h}{k_B T}
\]  

where \( \eta \) represents suspending fluid viscosity, \( V_h \) refers to hydrodynamic volume of the particle, \( k_B \) is the Boltzmann constant, and \( T \) is temperature. Dependence of this time constant on effective hydrodynamic volume, and therefore size and shape, highlights the importance of these two physical properties when IONPs are intended to act as flow mediators.

Equation 2-4 is valid for characteristic zero-field relaxation times, and effective relaxation rates through both physical rotation and internal reorientation become more rapid as the applied field increases (216, 217). Following previous studies that introduced corrections, effective field theory was utilized to capture the ferrohydrodynamic flow more accurately and consistently with the experimental data. Using this approach, an effective Brownian time constant is given by empirical expression derived from fitting a curve to data reported for large alternating magnetic fields (\( \alpha > 10 \)) (218):
\[
\tau_{B,H} = \frac{\tau_B}{(0.412\alpha^{0.809} + 0.143)}
\]

in which \(\tau_B\) represents the zero-field Brownian time constant obtained by Equation 2-4 and \(\alpha = \frac{\mu_0 mH}{k_B T}\) is the Langevin parameter defined by normalizing the energy scale of the magnetic moments in the external field to ambient thermal energy. Here, \(m\) denotes the magnetic moment of individual flow mediator, \(H\) is the external field magnitude, and \(\mu_0\) represents vacuum permeability.

Making use of the observed peaks in the flow versus frequency curves, it was possible to extract an effective hydrodynamic diameter for the MTB. Analogous study of IONPs was necessary for extracting meaningful hydrodynamic parameters for that system because the size of the aggregates changes over time. Because it relies on a process of aggregation, the structures that form depend on extrinsic conditions such as concentration in addition to intrinsic properties of the particles. As IONP clusters of different sizes form in the synthetic ferrofluid, its flow curve results from a distribution of dimers, trimers, quadruplets, and so forth (228, 229). However, here, we focus on extracting a single dominant hydrodynamic diameter associated with the maximum induced flow.

For MTB, the frequency corresponding to peak flow shifted upward with increasing field magnitude. Because of the complex geometry of the flow mediators, i.e. elongated cell body of a bacterium and IONP chain, effective hydrodynamic size can neither be easily measured nor extracted from analytical expressions. Using the peak frequency, which serves to indicate a timescale of relaxation, along with the average magnetic moment of single bacterium and multicore IONP extracted from the VSM data and theoretical considerations (Figure 2-4), we were able to correlate each effective hydrodynamic diameter with a peak frequency under different values of field magnitude for both MTB and IONP (Figure 2-5E). Comparing these values with the ones obtained from the experiments (Figure 2-13), the effective hydrodynamic diameter of MTB and a chain of IONP can be calculated. Figure 2-5F shows that the fitted hydrodynamic diameter of MTB lies between 1.8 – 1.9 \(\mu\)m for all of the three field magnitudes investigated. In contrast, the effective hydrodynamic diameter of the aggregates formed by the IONPs increases with increasing RMF magnitude.
In order to evaluate the validity of the extracted hydrodynamic diameter for MTB, the size distribution of the suspension was measured using a Multisizer (Figure 2-5G). Considering these values as the diameter of a sphere with equivalent volume, along with the TEM images, one can estimate dimensions of the bacterial body as shown in the subset of Figure 2-5G. Among different potential values for the hydrodynamic diameter of MTB, the equivalent diffusion coefficient is perhaps the most relevant. On the other hand, for IONP suspensions, the size distribution as measured by dynamic light scattering (DLS) in the absence of an applied field (Figure 2-5H) was not representative of the experimental condition, as it did not reflect the aggregation phenomenon. Formation of chains in the IONP suspension widened the size distribution, giving rise to aggregates with different dipole moments and hydrodynamic diameters. Comparing the value extracted from flow experiments with DLS data, the results were consistent with an average of approximately 3 multi-core IONP clusters per chain.
Figure 2-5: Quantification of ferrohydrodynamic flow of living and synthetic ferrofluids. A) An out-of-plane rotating magnetic field around y-axis was used to actuate MTB and IONPs in suspension confined in a customized PDMS well. B) Particle image velocimetry (PIV) results showing velocity magnitude and direction extracted from recorded actuation sequences. C) Magnitude of magnetically induced flow in MTB solution at different RMF frequencies under
three different field magnitudes. Each flow curve shows a maximum at a certain frequency called peak frequency. Error bars indicate 95% confidence intervals (N=7 separate experiments). D) Quantification of magnetically induced flow in IONPs suspension at three different field magnitudes. Error bars indicate 95% confidence intervals (N≥3 separate experiments). E) Variation of peak frequency as a function of hydrodynamic diameter at experimentally studied conditions for MTB and IONPs. F) Upper and lower bounds of extracted effective hydrodynamic size of MTB and IONPs aggregates. G) Size distribution of MTB solution obtained by a Multisizer and schematics of bacterial body with relevant dimensions for comparison with hydrodynamic diameter. H) Size distribution of IONPs suspension obtained by DLS and schematics of a chain showing different sizes associated with multicores IONPs.

2.4 Comparative performance of living and synthetic flow mediators

To identify the performance advantages offered by MTB, we determined figures of merit relevant to magnetically driven fluid transport applications and compared the two ferrofluids under investigation. Because homogenous flow is desirable, the velocity distribution serves as a useful parameter. PIV analysis of both samples in operating conditions that produced approximately the same average velocity across the region of interest (13.5 μm/s for IONP and 13.7 μm/s for MTB) revealed a more homogenous velocity distribution in the case of MTB, with relative standard deviation of 19.9% compared to 34.8% for the suspension of IONPs. To illustrate the velocity field uniformity, deviation of velocity from the mean value was calculated for both samples and plotted (Figure 2-6A and B).

The increased inhomogeneity in velocity found for the IONPs is attributable to broadly distributed sizes of aggregates and chains that form dynamically in the synthetic ferrofluid samples as a result of dipole-dipole interactions. In contrast, magnetosome chains are embedded in the cell body of the bacteria, mitigating magnetic interaction effects between MTB. From a transport perspective, the cell body of MTB acts to not only enhance the area of hydrodynamic influence, but also prevent them from interacting magnetically (Figure 2-5G), whereas IONPs form chains during magnetic actuation which elongate over time (Figure 2-5H). Although these chains increased the average hydrodynamic diameter of the colloidal components in synthetic ferrofluid, due to the size inhomogeneity, the control of flow became less homogenous and more time dependent. Moreover, these aggregation effects were partially irreversible, as indicated by size distribution analysis of flow mediators before and after 1 minute of actuation under a RMF of 12 mT (Figure 2-6C and D).

To facilitate comparison of MTB and IONP as functional materials for actuating flow, we introduce two figures of merit that consider the ratio of the induced flow velocity to the
amount of material employed for this purpose. Because number density of flow mediators correlates with magnitude of the induced flow, this parameter can be further adjusted depending on the application. However, the specific applications emphasized here require small volumes (microfluidic pumping and physiological administration), where it would be advantageous to use flow mediators with superior properties even if overall available quantities are lower. Nevertheless, scaled up culturing techniques have been reported for MTB (230). Here, to rule out the effect of number density and focus on intrinsic features of flow mediators, proposed figures of merit become large either when induced flow increases or the amount of required material decreases showing the intrinsic functionality of flow mediators. First, the magnitude of the generated flow was normalized by the volume fraction of magnetic material, i.e. iron oxide, emphasizing effects deriving from the magnetic material such as composition, size, and geometric configuration (Figure 2-6E). Induced flow of MTB solution per volume of magnetic material was more than two orders of magnitude higher than the corresponding value for IONPs. The pristine chains of magnetite particles in magnetosomes that promote magnetic order along their length and exhibit remanence when immobilized likely contribute the most to this advantage over synthetic counterparts (119, 120, 231, 232). Second, the flow magnitude was normalized by the total volume of functional material, i.e. either MTB cell bodies or IONP with all surfactant and functionalizing layers (Figure 2-6F). This highlights the role of hydrodynamic size, which is most relevant to coupling with surrounding fluid. Even though a considerably smaller volume fraction of MTB consists of magnetic material than the IONPs, normalizing induced velocities by the total volume of functional material reveals higher performance of MTB compared to IONP. This result underscores the functional benefits of the bacterial body, including its elongated shape and flagella for ensuring effective hydrodynamic coupling.
Figure 2.6: Comparative performance of MTB and IONPs as flow mediators. A,B) Distribution of velocity deviation from the mean value in the analysis domain as an indication of field uniformity for MTB and IONPs. Insets show velocity magnitude histograms corresponding to each sample. C,D) Size distribution of analysis domain before and after 1 minute of actuation for MTB and IONP obtained by image processing. Insets show the mean size of fluorescent NP aggregates before and after the actuation. Statistical significance was determined by Mann-Whitney test to compare the distribution (ns P=0.315 for MTB and ****P<0.0001 for IONPs). E,F) Induced maximum velocity per volume fraction magnetic material and per volume fraction net material inside the solution for living and synthetic ferrofluids.
2.5 Theoretical considerations of ferrohydrodynamic pumping

Using governing equations for ferrohydrodynamic pumping, magnetically induced fluid flow can be studied using computational platforms. Derived by Shliomis (233), a magnetization relaxation equation in time-varying magnetic fields can be written as:

\[ \frac{\partial \mathbf{M}}{\partial t} + (\mathbf{v} \cdot \nabla)\mathbf{M} = \omega \times \mathbf{M} - \frac{1}{\tau_{\text{eff}}} (\mathbf{M} - \mathbf{M}_{\text{eq}}) \quad 2-6 \]

Which explains the distribution and transport of magnetization vector \( \mathbf{M} \) inside the magnetic fluid where:

\[ \mathbf{M}_{\text{eq}} = M_s \left[ \coth(\alpha) - \frac{1}{\alpha} \right] \left( \frac{\mathbf{H}}{H} \right) , \quad \alpha = \frac{\mu_0 M_d V_p H}{k_B T} \quad 2-7 \]

represents the equilibrium magnetization vector under static fields. Under the application of time-varying magnetic fields, IONP magnetization continuously tracks the external field to align itself. However, as can be observed from equation 2-6, the magnetization vector \( \mathbf{M} \) does not necessarily align with the external field \( \mathbf{H} \) due to the opposing forces. Therefore, a lag forms between the magnetization vector and magnetic field vector, and dynamics of this alignment process are governed by Néel and Brownian relaxation mechanisms. While the former is associated with reorientation of magnetic moments within IONPs, the latter arises from physical rotation, and is coupled to rotation of fluid elements at their surfaces. Néel relaxation time for single domain particles showing superparamagnetic behavior is typically estimated by:

\[ \tau_N = \tau_0 \exp \left( \frac{KV_c}{k_B T} \right) \quad 2-8 \]

It should be noted that studied IONPs are considered as multi-core IONPs containing crystallites with diameters smaller than single domain size threshold (ca. 50 nm) which makes equation 2-8 a valid approach to calculate the Néel relaxation time constant. And for Brownian relaxation time:

\[ \tau_B = \frac{3\eta V_h}{k_B T} \quad 2-9 \]
The faster of the two mechanisms should dominate the alignment process, and the effective relaxation time constant for both processes acting in parallel is given by:

\[
\frac{1}{\tau_{\text{eff}}} = \frac{1}{\tau_B} + \frac{1}{\tau_N}
\]

For larger IONPs and aggregates, Brownian relaxation dominates, making fluid viscosity \( \eta \) and hydrodynamic diameter \( d_h \) crucial. Also, since ferrohydrodynamic flow relies on physical coupling between the particles and suspending liquid, we focused on the Brownian relaxation times for the purposes of this study. As shown previously (205, 207, 209, 223), maximizing the fluid flow is dependent on the relative magnitude of the actuation frequency and effective relaxation time constant of IONPs. Therefore, careful determination of Brownian relaxation time is essential for fluid flow applications.

Fluid motion under this condition is described using conservation of linear and angular momentum equations. For linear momentum it is given by:

\[
\rho \left[ \frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v} \cdot \nabla) \mathbf{v} \right] = -\nabla p + \mathbf{f} + 2\zeta \mathbf{v} \times \mathbf{\omega} + (\zeta + \eta) \nabla^2 \mathbf{v}
\]

where \( \mathbf{f} \) shows magnetic force density inside the ferrofluid and it is calculated as follows:

\[
\mathbf{f} = \mu_0 (\mathbf{M} \cdot \nabla) \mathbf{H}
\]

And conservation of angular momentum solves for spin velocity \( \mathbf{\omega} \):

\[
I \left[ \frac{\partial \mathbf{\omega}}{\partial t} + (\mathbf{v} \cdot \nabla) \mathbf{\omega} \right] = \mathbf{T} + 2\zeta (\mathbf{v} \times \mathbf{v} - 2\mathbf{\omega}) + \eta \nabla^2 \mathbf{\omega}
\]

Where \( \mathbf{T} \) represents the magnetic torque density obtained by:

\[
\mathbf{T} = \mu_0 \mathbf{M} \times \mathbf{H}
\]

As discussed earlier, moment \( \mathbf{m} \) of flow mediators lags behind the magnetic field vector \( \mathbf{H} \). Considering the definition of magnetic torque density, it is inferred that this lag gives rise to the magnetic torque \( \mathbf{T} \), the essence of torque driven flows. Therefore, the maximum fluid velocity, i.e. maximum hydrodynamic coupling, is achieved when magnitude of this torque peaks. In other words, out of phase component of the magnetization vector play more important
role here. One can obtain the following formula for the imaginary (out of phase) component of the complex susceptibility:

\[ \chi'' = \chi_0 \frac{\tau \Omega}{(\tau \Omega)^2 + 1} \]  

2-15

This explains the torque peak at \( \tau \Omega = 1 \). To establish a computational platform and conduct a proof-of-concept study on a previously studied geometry, the aforementioned ferrohydrodynamic governing equations have been implemented in COMSOL Multiphysics. The classical ferrohydrodynamic problem of pumping ferrofluid in channels is adapted from (207) and solved for different actuation frequencies as a test case. Figure 2-7A shows a traveling field inside the solution domain and illustrates how magnetization of flow mediators varies with respect to the external field as the field frequency changes. At relatively low frequencies, although magnetization is large, it remains almost in phase with the external field, leading to insignificant torque density. When the frequency matches the corresponding relaxation time constant, the out of phase component rises which is the direct consequence of having relatively large magnetization and sufficient lag at the same time. However, at higher frequencies, very low magnetization leads to a considerable drop in torque density despite an even larger lag between magnetization and magnetic field vectors. Magnitudes of induced flow and spin velocity are also given in Figure 2-7B, indicating the resultant fluid coupling under these conditions.
49

2.6 Computational modeling of spatially traveling and rotating magnetic fields

The magnetization relaxation equation introduced above can be written as a set of two simultaneous equations describing each component of magnetization vector:

\[
\frac{\partial M_x}{\partial t} + u \frac{\partial M_x}{\partial x} + v \frac{\partial M_x}{\partial y} = -\sigma_x M_y - \frac{1}{\tau} [M_x - M_{eq,x}] \quad 2-16
\]

\[
\frac{\partial M_y}{\partial t} + u \frac{\partial M_y}{\partial x} + v \frac{\partial M_y}{\partial y} = +\sigma_x M_x - \frac{1}{\tau} [M_y - M_{eq,y}] \quad 2-17
\]

In theoretical considerations, for the sake of simplicity and comparison to available data in literature, magnetostatic susceptibility is used to model the equilibrium magnetization, i.e. \( M_{eq} = \chi_0 H \), an assumption valid at low fields. One can also use the Langevin function to cover the whole range of magnetic field magnitudes. However, to model the flow of MTB solution and synthetic ferrofluid as accurately as possible, corrected VSM data are incorporated in
COMSOL Multiphysics as a function which is called by the software to calculate the equilibrium magnetization.

There are generally two approaches to model ferrohydrodynamic flow. The first approach, which is more common for the frequency ranges used for these applications, is modeling the magnetic field equations in the frequency domain and deriving time average force and torque densities which will be used in steady-state fluid flow equations. However, the second approach seeks to solve time dependent equations without time-harmonic assumptions. The choice between these two approaches is informed by two main considerations. Firstly, the actuation frequency determines the required time resolution, and in turn the necessary computational time. In addition, it indicates how large the time scale of field variations is compared to the time scale of fluid flow. Secondly, frequency domain formulation can only be applied to linear equations, which means for nonlinear materials the time-harmonic assumption is being violated. Taking these points into account, while proof of concept study in the context of theoretical considerations was done in frequency domain ($f = 50 \text{ kHz} \text{ and } \vec{M}_{eq} = \chi_0 \vec{H}$), time dependent simulation was carried out for both living and synthetic ferrofluids of interest ($f = 14 - 28 \text{ Hz} \text{ and } \vec{M}_{eq} = f(\vec{H})$).

In case of the first modeling strategy, which was utilized for the proof-of-concept study, assuming time-harmonic actuation in different forms, i.e. spatially traveling or uniform, one can rewrite the equations in frequency domain and solve them for phasors. For travelling magnetic field, actuation by current a current sheet was assumed in form of:

$$K = \text{Re}(J_s e^{i(\Omega t - kx)})$$  \hspace{1cm} 2-18

Which leads to the following equations for $\vec{M}_x$ and $\vec{M}_y$:

$$j\Omega \vec{M}_x - jku \vec{M}_x + v \frac{\partial \vec{M}_x}{\partial y} = -\omega_2 \vec{M}_y - \frac{1}{\tau} [\vec{M}_x - \chi_0 \vec{H}_x]$$  \hspace{1cm} 2-19

$$j\Omega \vec{M}_y - jku \vec{M}_y + v \frac{\partial \vec{M}_y}{\partial y} = +\omega_2 \vec{M}_x - \frac{1}{\tau} [\vec{M}_y - \chi_0 \vec{H}_y]$$  \hspace{1cm} 2-20

While for RMF, actuation is spatially uniform and time harmonic external field was assumed:
\[
H = \text{Re}(\tilde{H}e^{i\Omega t})
\]

Giving the equations in frequency domain:

\[
j\Omega \tilde{M}_x + u \frac{\partial \tilde{M}_x}{\partial x} + v \frac{\partial \tilde{M}_x}{\partial y} = -\omega_z \tilde{M}_y - \frac{1}{\tau} [\tilde{M}_x - \chi_0 \tilde{H}_x]
\]

\[
j\Omega \tilde{M}_y + u \frac{\partial \tilde{M}_y}{\partial x} + v \frac{\partial \tilde{M}_y}{\partial y} = +\omega_z \tilde{M}_x - \frac{1}{\tau} [\tilde{M}_y - \chi_0 \tilde{H}_y]
\]

Above equations were incorporated in COMSOL Multiphysics by the PDE Interface and defining the corresponding coefficients based on the derived equations.

### 2.7 Computational modeling of ferrohydrodynamic flow

As discussed in the Section 2.5, ferrohydrodynamic fluid flow was modelled by applying conservation of both linear and angular momentum for the fluid domain which solve for linear and spin velocities respectively. Considering 2D fluid flow, conservation of linear momentum for both \(x\) and \(y\) components are given by:

\[
\rho \left( \frac{\partial u}{\partial t} + u \frac{\partial u}{\partial x} + v \frac{\partial u}{\partial y} \right) = -\frac{\partial p}{\partial x} + \mu_0 \left( M_x \frac{\partial H_x}{\partial x} + M_y \frac{\partial H_x}{\partial y} \right) + 2\zeta \left( \frac{\partial \omega_z}{\partial y} - \frac{\partial \omega_y}{\partial z} \right) + (\zeta + \eta) \left( \frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} \right)
\]

\[
\rho \left( \frac{\partial v}{\partial t} + u \frac{\partial v}{\partial x} + v \frac{\partial v}{\partial y} \right) = -\frac{\partial p}{\partial y} + \mu_0 \left( M_x \frac{\partial H_y}{\partial x} + M_y \frac{\partial H_y}{\partial y} \right) + 2\zeta \left( \frac{\partial \omega_x}{\partial z} - \frac{\partial \omega_z}{\partial x} \right) + (\zeta + \eta) \left( \frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2} \right)
\]

Note that second term on the right hand side of the equations represent the magnetic torque density and third terms are a contribution from the spin velocity of particles in the fluid. Conservation of angular momentum in terms of spin velocity is as follows:
\[
I \left( \frac{\partial \omega_z}{\partial t} + u \frac{\partial \omega_z}{\partial x} + v \frac{\partial \omega_z}{\partial y} \right)
= \mu_0 (M_x H_y - M_y H_x) + 2\zeta \left( \frac{\partial v}{\partial x} - \frac{\partial u}{\partial y} - 2\omega_z \right)
+ \eta' \left( \frac{\partial^2 \omega_z}{\partial x^2} + \frac{\partial^2 \omega_z}{\partial y^2} + \frac{\partial^2 \omega_z}{\partial z^2} \right)
\]

Here, first term on the right hand side is the magnetic torque density.

Again, these equations are solved in a time dependent manner for both samples without any further assumptions. However, in the proof-of-concept study, above equations are solved under steady state condition. Therefore, using this time average modeling approach, transient terms vanish, and force and torque densities will be rewritten in time average form:

\[
\langle f_x \rangle = \frac{\mu_0}{2} \text{Re} \left( -jkM_x H_x^* + M_y \frac{\partial H_x^*}{\partial y} \right)
\]

\[
\langle f_y \rangle = \frac{\mu_0}{2} \text{Re} \left( -jkM_x H_y^* + M_y \frac{\partial H_y^*}{\partial y} \right)
\]

And

\[
\langle T_z \rangle = \frac{\mu_0}{2} \text{Re} (M_x H_y^* - M_y H_x^*)
\]

For both strategies, conservation of linear momentum is solved using Laminar Flow Interface of COMSOL Multiphysics in which magnetic force density and spin contribution were applied as volume forces. Also, the PDE Interface was employed to solve the conservation of angular momentum.

2.7.1 Deriving simplified definition of complex susceptibility

Neglecting inertial terms in magnetization equations in frequency domain, they can be simplified to:

\[
j\Omega \bar{M}_x = -\omega_z \bar{M}_y - \frac{1}{\tau} [\bar{M}_x - \chi_0 \bar{M}_x^*]
\]

\[
j\Omega \bar{M}_y = +\omega_z \bar{M}_x - \frac{1}{\tau} [\bar{M}_y - \chi_0 \bar{M}_y^*]
\]
Multiplying both sides by $\tau$ and rearranging the equations lead to:

\[(j\tau\Omega + 1)\hat{M}_x = -\tau \omega_2 \hat{M}_y + \chi_0 \hat{H}_x\]  

2-32

\[(j\tau\Omega + 1)\hat{M}_y = +\tau \omega_2 \hat{M}_x + \chi_0 \hat{H}_y\]  

2-33

Assuming negligible spin terms and inertial forces, relation between the magnetization and magnetic field under time-varying fields (complex susceptibility) can be derived from equation 2-6 under time harmonic actuations:

\[(j\tau\Omega + 1)\hat{M}_x = \chi_0 \hat{H}_x\]  

2-34

\[(j\tau\Omega + 1)\hat{M}_y = \chi_0 \hat{H}_y\]  

2-35

The above equations again can be written in vector form:

\[
\vec{M} = \frac{\chi_0}{(1 + j\tau\Omega)} \vec{H} \rightarrow \hat{M} = \frac{(1 - j\tau\Omega)\chi_0}{1 + (\tau\Omega)^2} \hat{H}
\]  

2-36

Finally, complex susceptibility under this specific condition containing both real (in phase) and imaginary (out of phase) components is given by:

\[
\vec{M} = \chi \vec{H} = (\chi' - j\chi'')\hat{H} \rightarrow \chi' = \frac{\chi_0}{(\tau\Omega)^2 + 1}, \chi'' = \frac{\chi_0 \cdot \tau\Omega}{(\tau\Omega)^2 + 1}
\]  

2-37

However, definition of complex magnetic susceptibility given in equation 2-37 can be modified by the effective field theory as follows (218):

\[
\vec{M} = \frac{(1 - jk''\tau\Omega)\chi_1}{(\tau\Omega)^2 + 1} \hat{H}
\]  

2-38

Again, imaginary and real parts can be extracted:

\[
\vec{M} = \chi \hat{H} = (\chi' - j\chi'')\hat{H} \rightarrow \chi' = \frac{\chi_1}{(\tau\Omega)^2 + 1}, \chi'' = \frac{k''\chi_1 \cdot \tau\Omega}{(\tau\Omega)^2 + 1}
\]  

2-39

Where following coefficients are proposed to take the field dependence behavior into account

\[
\chi_1 = \chi_0 \left[1 - \frac{0.0636\alpha^2}{1 + 0.18\alpha + 0.0659\alpha^2}\right]
\]  

2-40
\[ k'' = 1 + \frac{0.024\alpha^2}{1 + 0.18\alpha + 0.033\alpha^2} \]

The above equations are implemented as previously discussed in the computational platform.

2.7.2 Vortex viscosity of the suspensions with non-spherical particles

Both MTB and chains of IONPs are characterized by non-spherical shape effects, which is less studied in the relevant literature. Here, we used a mockup model to simply modify the well-known expression by Brenner \(^{15}\) for spherical particles, \(\zeta = \frac{3}{2} \eta \phi\), by adding a shape factor accounting for the effect of aspect ratios different than 1. For this purpose, a rotating object inside a liquid was modelled with different aspect ratios while keeping the area (volume fraction) constant under various rotational frequencies (Figure 2-8A). Defining vortex viscosity directly proportional to the ratio of exerted torque to the volume of rotating object, we were able to calculate the vortex viscosity associated with different aspect ratios relative to the object with aspect ratio of 1, i.e. sphere (Figure 2-8B). As expected, due to the low Reynolds regime, exerted torque was linearly correlated with the frequency and the same trend was observed under all rotating frequencies. Considering the obtained effective aspect ratio for MTB and IONP chain (Figure 2-5G-H), vortex viscosity was corrected for effect of non-spherical shapes.

![Figure 2-8: Effect of shape on the transferred torque. A) Four rotating objects with different aspect ratios and same surface area modelled computationally B) Reaction torques exerted on objects when rotating at different frequencies normalized by the torque applied to the object with aspect ratio of 1.](image)
2.8 Experimental and computational study of ferrohydrodynamic flow in confined channels

Because the envisioned applications of this transport mechanism entail confined spaces, such as blood vessels in case of drug delivery or channels in pumpless fluidic circuits, we further experimentally investigated the influence of boundary conditions on flow behavior in confined geometries with microscale dimensions. For this purpose, microfluidic chambers with a height of 100 µm (Figure 2-9A) were fabricated using conventional soft lithography techniques (Figure 2-12B). After filling these chambers with a suspension of MTB or IONPs, the center of the chamber was imaged under magnetic actuation via spatially uniform RMF. Here, the translation of magnetic torque into linear fluid flow relies on interaction with an upper or lower boundary. To investigate how the flow profile varied with position relative to two nearby parallel planes, we captured timed image sequences at different focal planes between the top and bottom walls.

Flow profiles of the MTB solution and suspensions of IONPs between parallel planes under this actuation condition are shown in Figure 2-9B and C, respectively. In both cases, an S-shape velocity profile was observed inside the channel during actuation. The mid-plane spatial symmetry of these boundary conditions resulted in a flow direction near the top wall that opposed the flow direction near the bottom wall, leading to a net cancellation of flow.

Our experimental characterization of flow in confined geometries, which revealed the importance of boundary conditions, motivated the development of a computational model capable of describing and predicting flow behavior under various magnetic actuation conditions and boundary geometries. Using the governing equations for ferrohydrodynamic pumping, we studied the magnetically induced fluid flow using the computational platform COMSOL Multiphysics. Here, a finite element method was employed, in which fluidic and magnetic governing equations were solved for variables of interest such as fluid velocity, magnetization, torque density, and a range of other parameters. This model enabled us to further analyze the flow at different conditions and gave insight into the comparative role of effective parameters. In order to validate our model, it was first applied to this empirically studied geometry Figure 2-9A). Spatially uniform RMF was incorporated as the externally applied magnetic field and the microscale parallel plane geometry was reconstituted for two-dimensional flow.
A robust model requires selecting suitable values for certain physical input parameters associated with magnetically driven fluid flow that have varied widely in literature (234, 235). Among them, perhaps the most disparate values have been adopted for spin viscosity, with reported theoretical and empirical values ranging over 10 orders of magnitude from $10^{-8}$ to $10^{-18}$ N s (236, 237). For this study, detailed analysis of the role of spin viscosity, $\eta'$, and vortex viscosity, $\zeta$, was carried out to determine suitable values for the systems under study and elucidate their role in the generated flow with determined $\eta' = 2 \times 10^{-14}$ N s and $\zeta = 1.3 \times 10^{-5}$ Pa s for MTB suspensions and $\eta' = 1 \times 10^{-11}$ N s and $\zeta = 6 \times 10^{-5}$ Pa s for IONP suspensions (Figure 2-8). Where possible, characterization techniques were employed to obtain other relevant physical properties such as shear and vortex viscosities (section 2.7.2). Briefly, an overall volume fraction of $5.8 \times 10^{-2}\%$ and shear viscosity of 5.5 mPa s were considered for the MTB, while the corresponding values for IONP suspension were assumed to be $2.5 \times 10^{-1}\%$ and 6.6 mPa s, respectively. Under these assumptions, the model was able to predict the magnetization distribution in channels and demonstrate how magnetization lags behind the magnetic field in response to time-varying actuation (Figure 2-9D). Magnetization of the samples was not uniformly distributed even though the external field was uniform, an effect due to the transport of magnetization by linear and spin velocities of the fluid. Noticeable differences in volume magnetization of the MTB and IONPs stems from higher concentration of flow mediators in synthetic ferrofluids. Numerical results also recapitulated flow profiles generated by the suspensions of MTB and IONP under actuation by RMF, as shown in Figure 2-9E and F. Although numerical results agreed well with experimental results for IONP suspensions, the model tends to underestimate flow velocities for MTB suspensions by approximately 40 to 50%. This discrepancy was mainly attributed to the limitations of the model in describing mechanical particle-particle interactions and taking into account the elongated shape of MTB, resulting in an underestimation of the MTB volume fraction. One can also see distribution of torque density across the channels for these torque-driven flows in Figure 2-9E and F, which represent relatively uniform distribution at the core of the channels.
2.9 Predictive modelling of control strategies for MTB for two application cases

Given the functional benefits that MTB exhibit, it is illustrative to consider in greater detail how they could be put to use in two distinct application cases. The examples examined here have been selected to reflect the two complimentary aspects of ferrohydrodynamic coupling—flow control of the surrounding liquid and directed transport of the magnetic mediator itself. Magnetically actuated microfluidic flow is a justifiable alternative to methods such as electroosmotic pumping in cases where exposing a fluid and its contents to strong electric fields could damage them, as with biomolecules or cells (238). Magnetically influenced delivery of therapeutic agents is a topic of perennial interest that still faces challenges to practical use at the scale of a human (20). The actuation method described here is unique, based on scalable magnetic torques and enhanced by properties of MTB. To devise advantageous and feasible control strategies, we have combined our experimentally validated computational model with predictions of realistic magnetic stimuli.
The first application considers the use of MTB suspensions to achieve well-controlled, localized fluid flows in microfluidic chips. Although net torque density is produced under actuation by a spatially uniform RMF, net flow cancels under boundary conditions of geometric symmetry, as shown in Figure 2-10. One strategy to produce directional flow is to break this symmetry magnetically with a non-uniform RMF, an idea that has been explored using traveling magnetic fields (207, 209). Here we considered using a non-uniform RMF with a gradient perpendicular to the direction of flow that preferentially increases MTB concentration at an actuation surface in order to intensify its influence on flow (Figure 2-10A). Using our established computational model to study the effect of different field gradient magnitudes on the net fluid flow inside a channel, we found that the desired redistribution of torque density across the channel becomes more pronounced as the magnitude of the gradient increases (Figure 5B). The net flow that results, averaged over a cycle, scales nearly linearly with the gradient magnitude over the range of feasible gradients (Figure 2-10B). Although flow varies throughout the cycle under this form of magnetic stimulus, the approach is still useful for applications with timescales larger than the period of rotation, a fraction of a second. Furthermore, the proposed gradient would be expected to draw MTB toward a boundary more strongly than predicted by the model, which assumes of uniformly distributed MTB for the sake of isolating the effect of locally controlled torque density and producing a conservative prediction of induced velocity. The feasibility of producing rotating fields with adequate gradients was studied by designing a realistic device for the purpose, sketched in Figure 2-10C and described in the following section 2.9.1.

The second control strategy is designed to produce spatially selective actuation of bacteria, regardless of their direction of transport. It is particularly desirable to pinpoint externally powered locomotion for drug delivery at a remote site such as a deeply seeded tumor, while avoiding dispersal into surrounding healthy organs and tissues. This can be accomplished by superimposing a nonuniform magnetostatic field with the RMF, analogously to the selection field applied in magnetic particle imaging and drug release triggered by hysteresis. The combined field ensures that effective actuation takes place only at points where the magnetostatic field approaches zero (239–241). Outside the zero point in the selection field, the magnetostatic field locks the magnetic moments of the MTB, ensuring no substantial magnetic torque (Figure 2-10D). Using the computational model, the principle was demonstrated by considering the effect of two permanent magnets located at top and bottom of a domain with
MTB in three hypothetical parallel channels positioned differently with respect to the magnets. While the torque density remained relatively high and quite stable in the zero-field area, the other channels were characterized by oscillatory and low magnitude torque density (Figure 2-10E).

To investigate the resolution of this approach when applied at length scales relevant to a human, the average torque density was calculated for channels exposed to different uniform magnetostatic fields. Our analysis showed that static fields as low as about 10 mT were sufficient to extinguish actuation of MTB, regardless of whether the magnetostatic field was oriented parallel or perpendicular to the channel (Figure 2-10E). The strength of this effect, which would be sufficient for centimeter resolution for gradient fields ten times weaker than those employed for magnetic particle imaging (on the order of 10 T/m) (242), is ultimately attributable to the high magnetic moment of stable chains contained within MTB. Figure 2-10 depicts a sketch of the envisioned application case, in which MTB actuation is selectively confined to the vicinity of a 1 cm diameter tumor. Details of the accompanying selection field can be found in the following section 2.9.2. Deeper analysis of this actuation scheme is presented in chapter 4 of the thesis.
2.9.1 Design characteristics of rotating gradient field for microfluidic pumping

The purpose of the setup represented in Figure 2-10C is to generate directional flow with spatially nonuniform rotating magnetic fields that draw MTB toward the upper boundary of a device while simultaneously coercing rotation. To verify the feasibility of producing this kind of magnetic stimulus and to study the attainable range of gradients, we developed a design with a working volume of 10 mm width and at least 5 mm height. The device features a gapped, soft
ferromagnetic core consisting of Permendur, an alloy selected for its high permeability and saturation flux density, although it may be possible to adapt the design for more economical materials. The geometry of the core is intended to offer independent control of the horizontal and vertical components of the field generated in the working volume via regulating current to two sets of copper windings. The first winding generates the vertical component of the field and encloses the center extension of the core. The second generates the horizontal component and is distributed between the right and left sides, which are wound in the same rotational sense and wired in series. The symmetry of the flux path ensures the flux generated by either of these windings should lead to net cancellation of voltages induced in the other coil, largely eliminating inductive crosstalk and allowing for independent driving with the appropriate phase shift to produce a rotating field. Performance of the system was studied for these two inductively orthogonal modes using 3D model geometry in COMSOL Multiphysics Magnetic Fields interface.

The core is designed to place the working volume in the fringing field of the air gap of a closed flux path, ensuring a relatively uniform field in the horizontal direction and a strong gradient in the vertical direction. If the geometry around the gap is kept constant and the cross-sectional area of other parts of the core are wide enough to avoid saturation, details of the core geometry away from the gap have a small impact on the field. The version shown in Figure 2-10C has incorporated a winding window sufficient to limit current density to less than 3 A mm$^{-2}$, depending on packing fraction of the wire. Such a design could be studied and modified for various expected operation times, improved waste heat rejection, increased efficiency, and tailored form factors for particular applications. The design shown in the figure is meant to be a realistic representation of a prototype version of such a device.

2.9.2 Design characteristics of selection field for drug delivery applications

In order to provide a working volume in which a magnetostatic gating field is superimposed on an RMF, the setup shown in Figure 2-10F was designed. It consists of two opposing pairs of air core Helmholtz coils and two sets of large permanent magnets. The Magnetic Fields interface of COMSOL Multiphysics was employed to study this setup, for which permanent magnets with a remanent magnetization of 1.4 T placed in opposing directions were modeled by two cylinders (30 cm diameter, 30 cm length) in a 2D axisymmetric geometry (Figure 2-11A). Torque density was mapped to the modeling domain using the curve shown in
Figure 2-10E and assuming a 40 cm diameter sphere as the main working volume outside of which RMF decays to zero. An actuated area of roughly 3 cm in diameter was achieved using this selection field (Figure 2-11B). The geometry is intended as a basic representation of a selection field, and alternative arrangements of permanent magnets could be used to improve the form factor or further restrict the selection point. Positional reconfigurations of the permanent magnets or DC offset currents in the coils that generate the rotating magnetic field could be used to control the position and size of the selection point.

![Figure 2-11: Proposed selection field generator. A) Distribution of magnetostatic field generated by two permanent magnets to provide selection field modelled in a 2D axisymmetric geometry. B) Variation of magnetic flux density along the centerline connecting two permanent magnets. Grey area shows the region in which the bacteria would be actuated.](image)

### 2.10 Conclusion

This work sheds light on the potential for MTB, bacteria with endogenous magnetic material, to act as mediators of magnetically induced fluid flow for pumping and transport. An integrated magnetic manipulation and imaging platform enabled us to quantify magnetically generated flow by MTB under RMFs and compare this living ferrofluid to a synthetic ferrofluid (IONPs) as a benchmark. The performance benefits offered by MTB indicated by PIV analysis included a more homogenous flow field in contrast to IONPs that underwent aggregation resulting in comparatively heterogeneous flow. Homogeneity is a relevant consideration for robust controllability and reliability. Moreover, figures of merit defined by normalizing the induced flow velocity indicated marked improvement of performance of the MTB over the IONPs, whether considered in terms of volume of magnetic material or net volume. This
suggests that both the magnetic and hydrodynamic properties of MTB contribute to their enhanced performance as a flow mediator.

Induced flows were then studied in confined geometries like those in end-use applications to elucidate the influence of boundaries, and to develop and validate a computational model. This model, combined with the favorable properties of MTB for flow actuation, enabled us to predict two realistic control strategies. The first introduced a spatial gradient to the applied rotating field to ensure net flow despite symmetric boundaries. The second applied a magnetostatic selection field to limit actuation to desired volumes.

While our experiments were performed at timescales far shorter than the reproductive lifecycle of MTB and the actuation scheme does not involve flagellar propulsion or activity of MTB themselves, the ability to self-replicate is a notable feature of MTB in certain applications. For example, in the context of bacterial cancer therapy, where bacteria are used as vehicles for the delivery of toxins (101, 162, 177), the local amplification of concentrations is central to the approach. Conversely, self-replication can be exploited merely as a convenient means to produce the advantageous physical properties of MTB, and sterilized swarms could be exploited in applications where continued replication is undesirable. Techniques such as microfluidic sorting and chemical mutagenesis followed by magnetic ratcheting could be employed to further enhance and standardize the magnetic properties and homogeneity (243, 244). Using MTB under RMFs as living functional materials holds promise for overcoming limitations associated with synthetic ferrofluids in transport applications such as drug delivery and pumpless microfluidic circuits.

2.11 Materials and methods

*Wireless Magnetic Manipulation Setup:* An arbitrary magnetic field generator consisting of eight electromagnets was used to apply rotating magnetic fields (Figure 1) (245). The system was mounted on the stage of an inverted microscope (Nikon Ti Eclipse) to make an integrated magnetic manipulation and imaging platform. Samples were positioned between the objective lens and a hemisphere formed by the ends of eight coils.

*Custom-made PDMS Wells and Chambers:* Conventional soft lithography was used to make customized wells and chambers out of polydimethylsiloxane (PDMS). A blank silicon wafer was utilized for ring preparation. The master for microfluidic chambers were created.
using photolithography of 100 μm thick negative photoresist (SU-8, MicroChem) atop a silicon wafer. Briefly, PDMS elastomer base and curing agent (SYLGARD 184 Silicone Elastomer Kit, Dow Corning) were mixed with the weight ratio of 10:1. The mixture was degassed and poured onto the corresponding silicon master. Following a brief additional degassing step, the mixture was cured at 70°C for at least 3 hours.

For custom-made PDMS wells, two different punchers (8 mm and 16 mm) were used to cut for inner and outer diameter of the ring. For microfluidic chambers, the cured PDMS slab was cut into desired pieces followed by punching inlets and outlets using biopsy punchers. All PDMS devices were bonded to glass coverslips following plasma treatment for 1 min. The final PDMS ring and microfluidic chamber is depicted in Figure 2-12B.

**Experimental Fluid Flow Analysis:** Red fluorescent NPs used for flow tracing (FluoSpheres carboxylated microspheres, 0.2 mm; excitation/emission, 580/605 nm) were added to all MTB and IONP suspensions at a concentration of 0.5% by volume of the manufacturer stock solution. MTB used for these experiments were harvested after passage 7. Samples were transferred to the PDMS wells (to a depth of less than 1 mm) or devices and placed in the integrated magnetic actuation setup. Fluorescence time lapses were taken to capture fluid motion at each field magnitude and frequency, with a focal height of 20 to 40 μm above the glass in the center of the PDMS wells. Image sequences were then analyzed using particle image velocimetry (PIV) implemented in the MATLAB PIVlab toolkit, in which fluid velocity is calculated based on tracked displacement of NPs.

**MTB Culture Condition:** Cultures of Magnetospirillum sp. AMB-1 (ATCC® 700264™) were used for this study. Bacteria were routinely passaged after 7 to 10 days and grown using magnetic spirillum growth medium (MSGM, ATCC® Medium 1653). Cultures were kept at 30°C under anaerobic conditions in 15 ml tubes.

**Cmag and Optical Density Measurement:** The growth curve and magnetic responsiveness of the culture of wildtype MTB was captured using absorbance measurements taken using standard polystyrene cuvettes in a Tecan Spark plate reader. Optical density (OD) of the solution at 600 nm was measured under no external field to obtain MTB concentration with a calibration curve. OD values were then measured under exposure to magnetic fields parallel and perpendicular to light path, using the fringing field of a small NeFeB bar magnet on top of the cuvette (Figure 2-2B). The Cmag value was calculated based on the ratio of the OD under
different field orientations to measure the bulk magnetic responsiveness of the sample. $C_{\text{mag}}$ data are shown in Figure 2-2A. Based on these data, an optimal timeframe can be identified at which both concentration and magnetic responsiveness for normal culture are at desirable values.

**VSM Measurement:** Magnetic properties of 100 μl suspensions of both MTB and IONPs were measured with VSM (EZ-VSM, Micronsense). For MTB, the culture was spun down and resuspended in phosphate buffer saline (PBS) to obtain OD of 1.2 which is equal to $8.4 \times 10^8$ cells/ml. For synthetic ferrofluid, fluidMAG-PVA (Chemicell 4120-5) was prepared at 1:10 dilution equal to 2.5 mg/ml. All samples were placed in NMR tubes shortened to 1 cm, capped, and affixed to custom modified glass sample rod with vacuum grease. Samples suspended in agar gel (4% by weight) were prepared by placing the solution atop 4 mg agarose powder, microwaving briefly in a shallow surrounding water bath to trigger gelation. VSM data for MTB and fluidMAG samples are given in Figure 2-2C and D, respectively.

**Rheological Measurements:** Shear viscosity of MTB and IONP suspensions were measured using an Anton Paar MCR 302 Modular Compact Rheometer with a water-cooled Peltier plate and a parallel plate geometry (20 mm diameter, Part No. 45950). Around 400 μl of the samples were pipetted onto the rheometer plate to fill the gap between the plates. Samples’ shear viscosity was monitored at constant temperature while shear rate was ramped up slowly from 0.1 to 100 s$^{-1}$.

**Modelling of Ferrohydrodynamic Flow:** Numerical simulation of the governing equations for magnetically driven fluid flow was implemented in COMSOL Multiphysics. An Eulerian frame was utilized, treating the whole fluid suspension as a continuum, and the effect of rotating flow mediators, whether IONPs or MTB, was incorporated by additional partial differential equations describing the magnetization of particles and fluid spin velocity \([198]\). Time-varying magnetic actuation was applied as the external magnetic field, which was coupled to fluid flow equations by magnetic force and torque density. For uniform RMF, the magnetization of ferrofluid was solved under the prescribed external field. Zero external pressure gradient was assumed for the fluid flow under both conditions and walls were defined using no-slip boundary condition for both linear and spin velocity.

**Flow measurement setup and fabricated devices:** Integrated magnetic manipulation is shown in Fig. S1A where the samples locate inside the working area of an array of
electromagnets. This setup provided uniform rotating magnetic field while enabling real time imaging of the samples at the same time. A customized PDMS ring and microfluidic chamber were used to confine the solutions with one and two walls respectively. While 75 μl of the liquid was used the PDMS ring, around 20 μl of the solution was enough to fill the microfluidic chamber. Both devices filled with colored fluid are shown in Fig. S1B.

Figure 2-12: Experimental setup for the ferrohydrodynamic studies. A) Integrated magnetic manipulation and imaging setup. B) PDMS ring as a customized well to confine the suspensions having only one wall at the bottom (left) and microfluidic chamber with 100 μm height to confine the suspensions by two walls one at the top and one at the bottom.

Peak Frequency Fitting: Determination of effective hydrodynamic size under actual operating conditions is challenging for the flow mediators used in this study for two main reasons. The first issue, most applicable in the case of IONPs, is the dynamic formation of
chains and aggregates that do not occur during other forms of characterization. The second issue comes from the relatively complicated shapes of flow mediators, implying that the hydrodynamic properties are best probed empirically. Knowing effective hydrodynamic size of the flow mediator is important for understanding their performance and helps to determine optimal operating conditions. Therefore, theoretical peak frequencies were calculated using the moment of individual flow mediators and as a function of effective hydrodynamic size.

\[\text{Figure 2-13: Estimation of hydrodynamic diameter for living and synthetic flow mediators. Empirical maximum flow frequencies are shown, along with bounded predictions for corresponding hydrodynamic diameter based on the model for relaxation time.}\]

![Graph showing estimated hydrodynamic diameters and empirical flow frequencies](image-url)
3 Magnetically enhanced local transport in tissue models

Sections 3.3, 3.5, and 3.8 of this chapter are adapted from (246), original draft of which was written by Tinotenda Gwisai followed by review and editing contributions from all the co-authors. The author acknowledges the contributions from Tinotenda Gwisai and declares that the in vivo study was performed in direct collaboration with her. Furthermore, Tinotenda Gwisai conducted the histological studies of the tumors harvested from the mice after the intervention. The author also acknowledges the help of Daphne Asgeirsson for taking the SEM images.

3.1 Introduction

Bacteria as living microrobots are equipped with onboard sensing and self-propulsion enabling them to target tumors in vivo (72). Different strains of Clostridium, Salmonella, Listeria, Escherichia, and Bifidobacterium have already been tested clinical trials (143, 247), and Bacillus Calmette–Guérin (BCG), an attenuated strain of Mycobacterium tuberculosis, is clinically approved for use in bladder cancer (71, 143). Nevertheless, inconsistent results in terms of tumor colonization and potential toxicities associated with their interactions with the immune cells have delayed translation of these living therapeutics in the clinic (100, 185). External control can offer a means to rapidly enhance tumor accumulation by applying forces that exceed propulsive forces intrinsic to the bacteria. Among different energy sources, magnetic fields can penetrate deep into the body without any absorption from the tissues. As a result, the innate responsiveness of MTB can be used to realize the goal of enhanced transport assisted by external magnetic fields through merging the benefits of bacterial cancer therapy and externally controllable microrobots.

Detailed analysis of the magnetic and hydrodynamic properties of MTB revealed their great promise as flow mediators (128). Magnetic order along the chain and their elongated body shape render them ideal microscale torque actuators. As a result, in addition to enhancing the convective transport, the torque driven mechanisms can further be explored for overcoming biological barriers. In our group, this capability of the bacteria was confirmed with experimental
evidence from in vitro assays (246). When exposed to RMF, MTB succeeded in translocating across endothelial monolayers cultured on transwell membranes in amounts exceeding the actuation by directing magnetic fields (DMF). Furthermore, RMF significantly increased accumulation of bacteria in tumor spheroids resulting in robust colonization compared to control samples.

Here, we try to elucidate the transport mechanism behind crossing endothelial barriers and tissue matrices. To this end, we establish a computational platform for studying the dynamic behavior of MTB as individual microrobots. Such a modeling strategy is required for study of bacteria when they undergo dilution in the bloodstream within a few minutes following injection. This platform together with the previously established model for swarm effects provide a broader understanding of the in vivo journey of intravenously infused MTB. We also expand the findings on overcoming the physiological barriers by studying the penetration of MTB into collagen gels as ECM-mimicking network. Following the study of transport in the hydrogels, a more sophisticated model including endothelial barriers and red blood cells is developed to test the performance of the transport mechanism under physiologically relevant conditions. The complementary role of the experimental and numerical models can be utilized for optimizing their targeting and delivery performance in the presence of biological barriers.

The current chapter ends with the results of an in vivo pilot study informed by the findings of the in silico and in vitro analyses.

Figure 3-1: Overcoming biological barriers by MTB exposed to RMF actuation. A) RMF outperforms DMF in helping bacteria cross endothelial barriers. Transwell assay with endothelial monolayer confirmed torque driven enhanced transport of bacteria. B) RMF results in robust colonization of the tumor spheroids by MTB. Clusters of bacteria are mostly present in the actuated spheroid following 5 days of incubation after the experiment.
3.2 Dynamic behavior of MTB as individual microrobots

Detailed understanding of the transport mechanisms realized by the magnetic guidance of MTB requires study of their behavior at different scales. While ferrohydrodynamic pumping addresses the phenomena at the scales above of 10s of microns, a zoomed-in analysis of bacteria as individual microrobots resolves the interactions at the microscale. In 1976, Purcell described the motion of a single micron-size bacterium swimming in a low Reynolds number environment (248, 249). Reynolds number defines the ratio between inertial forces and viscous forces:

\[
Re = \frac{\rho V L}{\eta}
\]

Where V and L are relevant velocity and length scales, \(\rho\) represents the fluid density and \(\eta\) is the fluid dynamic viscosity. Inserting values corresponding to AMB-1 into this formula, i.e. 10s of \(\mu m/s\) for the velocity and 1 \(\mu m\) the length scale (250, 251), one can calculate \(Re \approx 10^{-5}\) for these microorganisms. Equations governing this flow regime are known as linear Stokes flow equations which are derived from removing the inertial terms from the Navier-Stokes equations (252):

\[
0 = -\nabla p + \eta \nabla^2 \mathbf{u}
\]

Which in 2D cartesian coordinates can be written as follow:

\[
0 = -\frac{\partial p}{\partial x} + \eta \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2}\right)
\]

\[
0 = -\frac{\partial p}{\partial y} + \eta \left(\frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2}\right)
\]

The main characteristics of this flow regime are instantaneity and time-reversibility (92)(73). In other words, any transient behavior only emerges from time-dependent boundary conditions and the fluid motion is not influenced by the past. This, in turn, gives rise to the reversible nature of the flow which means through reversing the boundary conditions, the initial state of the fluid can be recovered. Another important feature of the Stokes flow is the linearity (253). The direct outcome of this characteristic is that forces \(F_h\) and torques \(T_h\) exerted on a rigid body with an arbitrary shape can be defined as linear functions of fluid linear velocity \(v_p\) and spin velocity \(\omega_p\):

71
\[
\begin{pmatrix}
F_h \\
t_h
\end{pmatrix} = \begin{bmatrix} a & b \\ c & d \end{bmatrix} \begin{pmatrix} v_p \\ \omega_p \end{pmatrix}
\]

The matrix relating these two sets of variables is called the resistance matrix and depend on intrinsic geometric properties of the rigid body (254). Theoretical formula for hydrodynamic force and torque have been derived for spheres undergoing pure translational or rotational motion (255).

\[
\text{Pure translation } \to \mathbf{F} = -6\pi \eta \mathbf{u}
\]

\[
\text{Pure rotation } \to \mathbf{T} = -8\pi \eta a^3 \mathbf{\omega}
\]

As can be seen from the above equations, coefficients of proportionality depend on fluid viscosity and shape factors. This behavior can be generalized for arbitrarily shaped objects by defining linear and rotational drag coefficients. By knowing these hydrodynamic interactions with the surrounding liquid, and assuming a rigid dipole moment for MTB, one can analytically study the dynamic behavior of these bacteria under rotating fields in the absence of any linear velocity (132).

\[
I \frac{d^2\theta}{dt^2} + \gamma \frac{d\theta}{dt} - \tau_m = 0
\]

\(\theta\) is the angular displacement of the bacterium, \(\gamma\) represents the linear drag coefficient forming the hydrodynamic torque when multiplied by the angular velocity. The first term containing the moment of inertia \(I\) is negligible under the Stokes regime. Solutions for average angular velocity of bacteria \(\omega_p\) can be obtained as the function of RMF frequency \(\omega_r\):

\[
\omega_p = \begin{cases} 
\omega_r & \omega_r < \omega_c \\
\omega_r - \sqrt{\omega_r^2 - \omega_c^2} & \omega_r \geq \omega_c
\end{cases}
\]

Where \(\omega_c = mB/\gamma\) represents the critical frequency defined by the maximum torque achieved by the magnetic field. The solutions for \(\omega_p\) define two regimes under application of RMF. With \(\omega_r\) below \(\omega_c\), also known as the step-out frequency, bacterium follows the rotation of the field in a synchronous way while the magnetic torque fails to compensate for the hydrodynamic resistance as \(\omega_r\) exceeds \(\omega_c\) leading to an asynchronous motion (Figure 3-2A) (256). These characteristic behaviors can be used for validation of the developed numerical
models. Implementing the above equations in COMSOL Multiphysics for an entity with MTB properties yielded the expected results for a microrobot under rotating fields (Figure 3-2B). Further details of this computational platform are discussed in Section 3.3.

![Figure 3-2: Dynamics of microrobots under rotating fields. A) Magnitude of the RMF frequency \( \omega_r \) relative to the critical frequency \( \omega_c \) determines the response behavior of the microrobot. Below the critical frequency the motion is synchronous with the external field while above it the microrobot exhibits asynchronous response characterized by back-and-forth motion. B) MTB response under 12 mT RMF at different frequencies. The angular displacement over time is obtained by Incorporating MTB data into the governing equations and solving them numerically in COMSOL Multiphysics.](image)

### 3.3 Computational modeling of interaction with endothelial monolayer

Since the endothelium is the first biological barrier encountered following intravenous administration, transport across a 2D endothelial cell monolayer was modeled. Given the prominent effect of RMF on MTB transport across robust cell barriers, we sought to understand the main mechanism driving enhanced translocation using a computational model in COMSOL Multiphysics. Finite Element Method (FEM) was employed to solve the governing equations using COMSOL Multiphysics. The cell monolayer was modeled in 2D featuring a few adjacent endothelial cells forming a sealed barrier between the upper and lower compartments. Endothelial cells were modeled as hyperelastic material with shear modulus of 1 kPa (257, 258). The dimensions of the cells in the monolayer was adopted from Arefi et al. (258). Considering the relative stiffness of gram-negative bacteria compared to endothelial cells, MTB were treated as rigid bodies except for the investigation of the force-driven migration pathways. Reported values for the elastic moduli of gram-negative bacteria range from 10s of kPa to 100s of MPa. For this specific study, we assumed Young’s modulus of 1 MPa for the bacteria which is in line with most reported values (259–261). MTB were modeled as ellipsoids initially present in the
upper compartment possessing a rigid dipole moment along their long axes. TEM images along with multisizer data were used to estimate the average size of a single bacterium. Assuming 0.45 um for the short axis and using the diameter of equivalent volume from multisizer measurements, the long axis of MTB was estimated to be approximately 1.8 um.

Motion of micron-sized objects in a fluid close to a wall is well studied as surface walkers under low Reynolds number flows (254, 262, 263). The free body diagram of single bacterium illustrates the balance of acting forces and torques in such an environment:

\[
\sum \vec{F} = 0 \Rightarrow \vec{F}_H + \vec{W} + \vec{F}_C = 0
\]

\[
\sum \tau = 0 \Rightarrow \tau_M + \tau_H + \tau_C = 0
\]

\(F_H\) indicates the viscous drag, \(W\) is the gravity force, and \(F_C\) is the reaction force from the substrate including both normal and frictional components. A mass density of \(\rho = 200 \text{ kg/m}^3\) was assumed for the bacteria to take the effect of buoyant force into account (264). In the conservation of angular momentum equation, \(\bar{U}_M\) represents magnetic torque, \(\bar{U}_H\) is hydrodynamic resistant torque, and \(\bar{U}_C\) indicates the torque coming from the interaction with the substrate and is given by:

\[
\tau_C = rF_f
\]

where the distance from the axis of rotation \(r = 0.9 \mu m\) and \(F_f\) is the friction force.
Magnetic torque arising from the lag between the magnetic dipole moment of MTB and external field is given by:

\[ \tau_M = mB \sin \theta \]  \hspace{1cm} (3-13)

where \( m \) is the magnetic dipole moment of MTB which is estimated from VSM measurements to be approximately \( 6 \times 10^{-16} \) A.m\(^2\) \((128)\), \( B \) represents the external magnetic field, i.e. 20 mT, and \( \theta \) is the phase lag between two vectors.

Stokes flow theorem correlates hydrodynamic interactions in terms of linear and rotational viscous drag with corresponding linear and angular velocities:

\[ \vec{F_H} = \gamma \vec{v} \]  \hspace{1cm} (3-14)

\[ \vec{T_H} = \alpha \omega \]  \hspace{1cm} (3-15)

where \( \gamma \) and \( \alpha \) are the linear drag coefficient and rotational drag coefficient, respectively. Analytical expressions and empirical data were used to estimate the values of these parameters which were set to \( \gamma = 7.1 \times 10^{-9} \) N.s/m and \( \alpha = 7.6 \times 10^{-20} \) N.m.s/rad in the simulations.

The velocity dependence of friction between two microscale objects has previously been reported \((265)\). This feature was incorporated into the friction model in which the slip and no slip regime was determined by a critical friction force depending on normal traction and velocity difference between two objects at the point of contact. Numerically, the penalty method was employed to resolve the mechanical contact which is based on insertion of a spring between two objects, only active in the case of compression.

Rigid Body node of the Solid Mechanics interface was used to solve the above governing equations. In order to avoid the computationally expensive fluid solid interaction simulation, hydrodynamic interactions were modeled as linear and rotational viscous damping acting on the rigid body.

### 3.4 Investigation of force-driven migration across monolayers

Bacteria rely on flagellar motion for propulsion within their surrounding environment. These forces, although strong enough for navigation and chemotactic migration in their natural
habitat, potentially limit their ability in crossing biological barriers inside the body. Knowing the average swimming velocity of bacteria, one can estimate the order of propulsive forces under the assumption of Stokes regime. Reported values of swimming velocity for strain AMB-1 range from 19 to 49 $\mu m/s$ \((250, 251)\) by which propulsive forces were approximated to be:

$$F_p = \gamma v \approx 0.2 \text{ pN}$$

On the other hand, externally applied magnetic fields are able to override the natural motion and bring about forces higher than those of propulsive alone. A simplified calculation of the force required to compensate the applied torque via RMF provides an order of magnitude estimate for magnetically induced forces. Taking the long axis of the bacteria as the arm of the moment, this force was estimated as follows:

$$F_C = \alpha \omega / r \approx 5 \text{ pN}$$

This indicates that potentially one order of magnitude higher energy levels are transferred with this actuation strategy.

Encouraged by the result of this theoretical analysis, we first explored the possibility of force-driven migration across the endothelial monolayers. In order for immune or cancer cells to pass through paracellular pathways, they need to overcome two types of resistant forces. First, squeezing between the endothelial cells exposes them elastic forces coming from the deformed cells. Another source of resistance is different types of endothelial junctions such as adherens junctions that are formed by transmembrane proteins.

To examine the elastic resistance faced by the bacteria, we used the in silico model discussed in the previous section. The cells were modeled as hyperelastic materials with a shear modulus of 1 kPa \((257, 258)\) and dimensions adopted from Arefi et al. \((258)\). The forces required for a single bacterium to pass through a passive junction where the boundaries of two adjacent cells were in contact was computed \((Figure 3-4A)\). Although such an idealized attachment might not exist, this geometrical configuration was employed to yield an order of magnitude estimate for the resisting force. The contact force profile for the y component demonstrated 10s of $pN$ values which is above the calculated range for the attainable values under RMF. This force is reported to be in the range of 10s nN for extravasation of the immune and cancer cells \((258, 266)\). Although the obtained value for the resistant force may overestimate the level of forces that this living microrobot encounters in vivo, the calculated
torque-induced force also represents an upper bound given the nature of the interactions with endothelial barriers. When rotating in a fluidic environment, the buoyant force and viscous resistance prevents full conversion of rotational energy to a contact force. Using the same model with a bacterium rotating at 14 Hz under 12 mT RMF, we showed that the contact force between the bacteria and cells are far from the calculated upper bound (Figure 3-4B). Contact force approaches the theoretical maximum when mass density increases or viscous resistance becomes lower.

Figure 3-4: Computational study of force-driven translocation across monolayers. A) Single bacterium with prescribed motion across the monolayer. Contact force resulted from this motion reflects the level of the resistance needs to be overcome for successful transmigration. B) Mechanical interaction of the single bacterium with the cells on the monolayer. Rotating nature of the motion leads to discontinuous contact with the cells. Lower level of contact forces are measured under rotation.

In addition to the above resistance, junctional complexes need to be removed for successful transmigration. For instance, vascular endothelial-cadherin (VE-cadherin), an endothelial cell-specific adhesion molecule is thought to play a key role in the extravasation of immune and cancer cells (267, 268). In those cases, contributions from biochemical signaling
and force-driven rupture of the bonds have been reported \((269, 270)\). As a result, even when the membranes of neighboring endothelial cells are not touching, transendothelial migration of bacteria requires overcoming this local barrier. Analyses of VE cadherin bonds at single molecule level has predicted 10s of pN forces required for breaking individual bonds \((267)\). Our estimates above show that, on average, torque-driven mechanical interactions generate forces that are an order of magnitude higher than those of MTB self-propulsion, yet lower than those required to squeeze through adjacent cells and break their junctional bonds.

### 3.5 Role of torque-driven motion on translocation

Torque exerted on the bacteria upon exposure to RMF results not only in forces that are applied on the cell surface, but also the generation of substantial translational motion. This suggests that the application of forces directly on an endothelial monolayer cannot be the chief mechanism responsible for enhanced translocation. As such, we sought to examine the influence of translational motion derived from torque-based actuation on bacterial translocation.

An endothelial cell monolayer was modeled with adjacent cells forming a sealed barrier between upper and lower compartments. Recent work has shown that dynamic mechanical processes within the endothelium result in gap formations that are independent of the influence of migrating cancer or immune cells \((271–273)\). Using this as a basis, stochastic opening of cell-cell contacts was incorporated into the model to account for the active mechanics of the endothelium. For each simulation, the gap lifetime was set to 160 s \((35)\) and a set of random parameters were generated to determine the gap size, which was within the range of 1.5 to 2.5 \(\mu \text{m}\) \((34)\). The overall simulation time was selected to encompass opening incidences for all gaps. MTB were modeled as rigid ellipsoids possessing a rigid dipole moment along the long axis. Dimensions of 0.45 and 1.8 \(\mu \text{m}\) were assumed for the short and long axis respectively \((\text{fig. S1})\) \((128)\). Hydrodynamic interactions were modeled as linear and rotational viscous damping acting on the rigid body.

To characterize the permeability of our modeled monolayer, simulations of passive diffusion of liposomes with a diameter of approximately 200 nm were performed and 5.9\% of the liposomes diffused into the lower compartment \((\text{Figure 3-5A})\). Having established the model, we proceeded to compare MTB transport under DMF and RMF \((\text{Figure 3-5B})\). The velocity profiles generated for the bacteria under RMF exhibited the well-studied characteristics of
surface walkers under low Reynolds number flows (59–61). The higher mass density of MTB with respect to the surrounding liquid gives rise to a terminal velocity of the bacteria which results in an offset in the y component. When traveling along the monolayer, there is discontinuous contact with the surface and the contributions from the x and y components of the velocity vary depending on whether the MTB is traveling downhill, uphill or is translocating. As anticipated, the contribution from the x component is minimal as the MTB passes through an opening, reflecting the lowered contact with the cell surface.

Our model showed that only 6.6% of MTB exposed to DMF crossed the barrier, compared to 20% of MTB exposed to RMF (Figure 3-5C). Under the static conditions of DMF, the bacteria only passed through the monolayer when initially located in close proximity to a cell-cell junction. In contrast, MTB under RMF translated along the monolayer which enabled the bacteria to explore the monolayer surface and pass through any gaps that formed between the cells. Overall, these findings strongly suggest that enhanced surface exploration resulting from torque-based translational motion is the dominant mechanism facilitating increased translocation of MTB.

Figure 3-5: Computational modeling of MTB-LP transport across endothelial monolayers. (A) Computational simulations of liposome diffusion across a modelled endothelial monolayer. Cells were modeled as hyperelastic materials and Stochastic opening of cell-cell contacts was incorporated into the model to account for spontaneous gap formation. (B) Simulations MTB transport under DMF and RMF across a modelled endothelial monolayer. MTB were modeled as rigid ellipsoids possessing a magnetic dipole moment. Velocity profiles show contributions of x and y components of MTB traveling downhill (1), uphill (2), and translocating through a cell gap (3). (C) Plot of the average amount of liposome diffusion and MTB translocation under DMF and RMF for 3 simulations relative to the starting concentrations.
3.6 Penetration into tissue-mimicking hydrogels

A microfluidic device mimicking the vascular channels and extracellular matrix (ECM) components was used as a physiologically relevant in vitro model to study MTB penetration into barriers presented by the ECM. Such studies require proper modeling of the liquid-tissue interface to yield results that are translatable to in vivo conditions. For this purpose, we adopted a compartmentalization technique called contact line pinning through which the difference between the critical advancing angle and receding angle is increased in order to strengthen pinning of a liquid to a surface. This goal is achieved by adding microfabricated ridges on the surface which help confine the collagen in the designated areas resulting in wall-less channels and blockage free interfaces. Two-layer deposition of a photoresist was required to produce these structures through photolithography techniques. Further information is available in the Materials and Methods section, 3.10.

Untreated PDMS shows hydrophobic surface properties (contact angle of 125° with DI water) which transitions to completely hydrophilicity after exposing the silanol groups on the surface following plasma treatment. It has been reported that after two weeks at room temperature PDMS recovers its hydrophobicity (contact angle of around 90°) and the difference between advancing and receding angles reaches a quasi-steady state 2-3 days post plasma treatment. Taken these observations into account along with ease of injection and avoiding bubbles inside the channels, 2-5 days after bonding PDMS to glass slides was set for the time window of performing the experiments.

The designed microfluidic device consisted of three channels, one in the center and two side channels, that intersect with two larger perpendicular chambers (Figure 3-6A). Ring-shaped small channels connected to the central channel and side chambers were added to differentially pressurize various compartments; but they were not employed for this set of experiments. Such a design provides the flexibility of confining collagen at different locations separated by contact lines. Here, to study penetration of MTB into the collagen network as the main constituent of ECM, the side chambers were filled with collagen and a suspension of GFP-expressing bacteria was introduced into the channels. TAMRA-labelled collagen enabled visualization of the successful patterning inside the chip (Figure 3-6B). The presence of microfabricated ridges was confirmed with the help of SEM images (Figure 3-6D and E).
height of these structures was approximately 15 μm which is equal to 10% of the total height of the features created from the photoresist.

Considering the settling velocity of bacteria and time span of the magnetic actuation, local concentration gradient was expected to be generated in the course of the experiment. Such a gradient would break the symmetry in favor of the wall effect from the bottom side leading to the creation of a dominant flow direction as opposed to the previously studied s-shape profile. As a result, penetration of the bacteria into the collagen compartments would become asymmetric. To account for this effect and also demonstrate the capability of the actuation scheme to spatially control the enhanced transport, alternating RMF, where the direction of the rotation was reversed every 7.5 min, was employed for these experiments. As such, the actuation period was divided into four parts which equally distributed the translational motion between the two interfaces (Figure 3-6C).
Chapter 3

Magnetically enhanced transport in tissue models

Figure 3-6: In vitro test platform for study of MTB penetration into ECM mimicking hydrogels. A) Schematic of the contact line pinning microfluidic devices exposed to the magnetic actuation. B) Compartmentalization of the bacteria and collagen gel through microfabricated contact lines. Suspension of GFP expressing bacteria (green) is introduced between two chambers filled with TAMRA labelled collagen (red). C) Alternating RMF for balanced penetration of the bacteria at both interfaces. Gravitational settling of MTB leads to formation of a concentration gradient and preferred flow direction over time.

Penetration of bacteria was represented with two parameters reflecting the net amount of bacteria inside the collagen and the migrated distance through this compartment. Since manual injection of MTB into the device resulted in variable initial accumulation in collagen, both penetration distance and accumulated bacteria were quantified relative to their corresponding values at time point zero. First, we exposed the samples to alternating RMF at 12 mT with different frequencies to study the dependence of transport on the actuation frequency. Quantification of the increased penetration distance exhibited a shift in optimal frequency towards lower values compared to fluid flow experiments where 14 Hz yielded the highest output (Figure 3-7A). The same trend was also observed in the amount of bacteria accumulated in collagen. Results indicated significant magnetically enhanced transport of
bacteria under frequencies below 10 Hz (Figure 3-7B). Such behavior was expected due to the higher resistance presented by the collagen network. This change in resistance would be translated into a higher equivalent viscosity of the surrounding medium which leads to the shift of the step-out frequency. In other words, a combination of three types of interactions playing a role in this transport mechanism determined the optimal actuation conditions. The first physical interaction occurs when the bacterium is fully surrounded by a liquid. Next, an interaction takes place at the boundary where intermittent contact with the gel is present. Lastly, the penetrated bacteria undergo the third type of interaction with the fibrous network which behaves like a porous material. This complexity underlies the lack of a smooth single-peak trend in frequency dependent behavior in comparison to ferrohydrodynamic pumping.

Next, we sought to better elucidate the effects of alternating RMF in comparison to constant RMF characterized by unidirectional actuation of MTB (Figure 3-7C). These experiments were conducted at 12 mT and at frequencies lower than 10 Hz where significant increase in the MTB penetration was observed. For constant RMF, the transport into the targeted interface was analyzed in addition to the average values of both interfaces. Interestingly, while the values for a single interface were comparable to the alternating RMF, averaged values for the constant RMF actuation strategy were similar to unactuated controls. These results implied that there could be a saturation level for continuous manipulation of bacteria at an interface which can potentially be due to blockage of pores available for translocation into the fibrous network. This is also accompanied by depletion of bacteria at the non-target interface which would result in lower penetration than in controls. Therefore, the benefits offered by application of the alternating RMF justified further use of this actuation scheme for subsequent experiments.

Next, we repeated the same set of experiments at a higher field magnitude of 20 mT. Following the mechanistic study of penetration at 12 mT, experiments at a higher field assisted in investigating whether similar trends to ferrohydrodynamic pumping would be present. Additionally, the finding from this experiment could be used to determine the actuation conditions for in vivo experiments where higher complexity of barriers demand stronger torques to achieve comparable results. Again, a similar shift compared to the corresponding ferrohydrodynamic results (24 Hz at 20 mT) was observed (Figure 3-7D). However, higher torque levels resulted in better performance at higher frequencies in comparison to results at 12 mT. As expected, a higher increase in the penetration distance (~150 μm) was achieved.
Analogously, more bacteria accumulated in the collagen at this higher field magnitude where actuation at 6 Hz and 14 Hz produced better transport characteristics (Figure 3-7E). Representative confocal images confirmed this magnetically enhanced transport of GFP MTB (Figure 3-7F). They showed symmetric, increased accumulation inside the collagen compartments. Taking these two findings together, 14 Hz was selected as the operating frequency for in vivo studies.

**Figure 3-7**: Magnetically assisted penetration of the MTB into collagen gels. A) Increased penetration distance over the course of the experiments (30 min) under RMF at 12 mT with different frequencies. B) MTB signal in collagen at the end of the magnetic actuation at 12 mT normalized by the initial time point. C) Comparison of MTB penetration under different actuation schemes. Targeted single interface at constant RMF exhibits similar values to the average of both interfaces under the alternating RMF. D) Increased penetration distance over the course of the experiments (30 min) under RMF at 12 mT with different frequencies. E) MTB signal in collagen at the end of the magnetic actuation at 12 mT normalized by the initial time point. F) Representative images of the bacteria before and after the magnetic actuation (scale bar 200 μm).
3.7 Transport characteristics in endothelialized channels

Following computational and experimental studies of MTB transport in the presence of different tissue barriers, we continued by integrating the previously studied barriers into the microfluidic platform. Also, the effect of red blood cells (RBCs) on MTB transport was studied as RBCs are the main constituent and the most abundant cell type in blood. The same devices as previously described were used for seeding the HMEC-1 cells to form an endothelial monolayer at the interface with the collagen (Figure 3-8A). To this end, a cell suspension at a concentration of $1\times10^8$ cells/ml was introduced into the collagen embedded devices. Following 3 days of culture a bacterial suspension was injected into the channels. This set of experiments were conducted at a lower MTB concentration compared to the previous experiments because of the observed saturation effect ($5\times10^8$ vs $9\times10^8$ cells/ml). To ensure the presence of a complete monolayer at the interface, cells were stained for VE-cadherin. Continuous expression of the VE-cadherin along both interfaces confirmed the existence of endothelial adherens junctions inside the monolayer (Figure 3-8B). Although the presence of a confluent monolayer at the bottom was not crucial for these experiments, microscopic visualization of these bonds was easier for the cells seeded at the bottom (Figure 3-8C).

For the actuation experiments, the prepared devices were exposed to RMF at 20 mT for 30 minutes. Since the bacterial concentration was almost half of the concentration used for the transport studies in collagen, alternating RMF with longer cycles (15 min) were applied. RMF actuation was performed at 6 Hz and 14 Hz as these resulted in better transport characteristics in the collagen hydrogels. Effects of both bacterial self-propulsion and magnetically enhanced transport, which are reflected in the control and the actuated samples respectively, were more pronounced here in the presence of endothelial cells (Figure 3-8D). This is a result of the presence of the monolayer at the interface which forms a tighter barrier for infiltration into collagen leading to lower initial accumulation following manual injection of the bacteria (Figure 3-8E). In the absence of RBCs, magnetic actuation at both 6 Hz and 14 Hz enhances the transmigration and transport to the collagen compared to the control. However, RBCs acted as a hemorheological barrier partially impeding the magnetically enhanced transport. Further control experiments such as transport in the presence of RBCs and without magnetic actuation of bacteria can help elucidate the contributions from different barriers.
3.8 Assessment of intratumoral transport of MTB in vivo

Motivated by the pronounced effect of RMF on MTB transport in vitro, we next sought to test whether our RMF actuation strategy also enhanced bacterial accumulation in vivo with a pilot study employing a syngeneic mouse model. BALB/c nude mice bearing subcutaneous MCF-7 tumors in one hind flank received intravenous injections of $1 \times 10^9$ MTB stained with a far-red proliferative dye (Figure 3-9A). The anaesthetized mice were placed either in the absence of magnetic actuation (control) or with tumors positioned in the workspace of a magnetic field generator applying a 20 mT RMF at 14 Hz for 1 h. For an applied field magnitude of 20 mT, uniform fields with negligible offset gradients are expected within the workspace of the field generator (Figure 3-9B), ensuring that observed effects on the accumulation of bacteria can solely be attributed to the rotational character of the applied field.
Following treatment, mice were returned to the cage for 24 h, after which the tumor and major organs were harvested to assess the distribution of the bacteria using a whole organ fluorescence scanner (Figure 3-9C). Fluorescence signal was detected in all tumors suggesting that the bacteria innately accumulate in tumors. Notably, this signal was 3.16-fold higher in mice with tumors exposed to RMF compared to unactuated controls (Figure 3-9D). Very low signals were detected in most of the major organs except for the liver, consistent with previous findings 24 h after intravenous administration of AMB-1 (274). Bacterial accumulation in the liver is expected to decrease over time, with full clearance by day 6 (274).

To assess whether the bacteria detected in the fluorescence scans were still viable, harvested tumors were homogenized and placed into MTB culture media. After 8 days, large, dark pellets that were magnetically responsive were present in all tubes, indicating that the tumor homogenates contained live MTB. $C_{mag}$ measurements were performed to quantify the magnetic properties of the cultures and all samples had values above 1, signifying magnetic responsiveness of the bacterial suspensions (Figure 3-9E).

Histological sectioning of the tumors was performed, with sections collected at a depth of approximately 1 mm in the tumor showing more pronounced accumulation at the periphery in both control and actuated samples (Figure 3-9F). Mean intensity distributions with increasing distance from the periphery for the tumor sections show that bacteria can be detected throughout the measured region, with higher overall signal from samples exposed to RMF compared to controls (Figure 3-9G). This result indicates higher penetration for RMF actuated tumors.

Transverse histology sections were also collected to assess the distribution of MTB with increasing depth within the tumor (Figure 3-9H). The mean intensity of each section was used to compile a fluorescence intensity distribution (Figure 3-9I). The intensity increases with increasing depth in the first 800 μm of RMF-exposed samples that were analyzed, whereas intensity decreases with depth in the controls. The average fluorescence intensity for consecutive transverse tumor sections showed that the overall fluorescence intensity, and thus the amount of MTB delivered in the actuated samples, is 2.1-fold higher than in the controls (Figure 3-9J). These results, combined with the findings from our in vitro analysis, demonstrate the potential of magnetic torque-driven control strategies for enhanced tumor accumulation.
Figure 3-9: Assessment of intratumoral transport of MTB in vivo. A) BALB/c nude mice bearing subcutaneous MCF-7 tumors in one hind flank were intravenously administered with $1 \times 10^9$ MTB stained with a far-red proliferative dye. The mice were anaesthetized for 1 h in the absence of exposure to magnetic actuation (control) or were placed on a magnetic field generator with tumors positioned in the workspace (RMF). Mice were returned to the cage for 24 h, after which the tumor and major organs were harvested for further analysis. B) Plots of expected field magnitude and gradients produced in the workspace of the magnetic field generator for an applied field of 20 mT in x. (C) Representative ex vivo fluorescence intensity images of major organs and tumors 24 h after injection of far-red stained MTB. D) Quantitative biodistribution from harvested organs and tumors ($n = 3$; mean ± SD; **$P < 0.01$, Student’s t-test). E) $C_{mag}$ values for homogenized tumors placed in liquid culture for 8 days ($n = 3$ control, $n = 4$ RMF; mean ± SD). F) Representative images of 10 μm histology sections which were sectioned at depth of approximately 1000 μm in the tumour. Cell nuclei were stained using Hoechst 33342 (blue). G) Mean intensity values with increasing distance from the periphery of the tumor sections ($n = 3$; mean ± SD). H) Representative transverse tumor sections. Cell nuclei were stained using Hoechst 33342 (blue). I) Plot of mean intensity for each traverse tumor section at 200 μm intervals. Values were normalized to overall minimum and maximum fluorescence intensity values. J) Summation of mean intensity values for consecutive traverse tumor sections ($n = 3$; mean ± SD).
3.9 Conclusion

This chapter elucidated the benefits of using MTB as living microrobots to locally enhance the transport to the tissues through external application of RMF. A computational model was developed to study dynamic behavior of individual bacteria under rotating fields. The same model indicated that the force-based mechanisms can hardly explain the translocation across endothelial monolayers. More detailed numerical study of actuated bacteria on monolayers revealed that enhanced surface exploration appeared to be mainly responsible for the transmigration.

Microfluidics platforms were then employed to study the penetration of MTB into collagen gels. Effective higher net resistance arising from interaction with collagen fibers was reflected in the shift of optimal actuation frequency towards lower values. A more physiologically relevant in vitro model in form of endothelialized channels better highlighted the effect of the magnetically enhanced transport into tissue-mimicking compartments when a tight barrier is present.

The findings from the computational and experimental studies were incorporated in the final in vivo experiments. Biodistribution studies showed selective MTB accumulation in the tumor which was further enhanced by performing RMF actuation locally at the tumor site. These results altogether provided evidence for the merits of magnetically responsive organisms in the context of drug transport. Therefore, developing strategies for RMF-based magnetic actuation at larger scales can pave the way for clinical applications of such living microrobots.

3.10 Materials and Methods

**Microfluidic device preparation:** All microfluidic chips were fabricated using polydimethylsiloxane (PDMS) according to standard soft lithography protocols. Microfluidic patterns including channels and wells were designed in AutoCAD. Final designs were transferred into GerbTool 16.9 to adjust transparency of different areas prior to printing them on the photomask. Photolithography techniques were then employed to make the master molds out of 4-inch silicon wafers. Briefly, wafers were spin-coated by a ~130–μm thick layer of highly viscous negative photoresist (SU-8 3050, MicroChem). First layer underwent the UV exposure through a foil mask containing patterns without the contact lines. Following sufficient baking and subsequent resting at the room temperature, a low viscosity negative photoresist
(SU-8 3010, MicroChem) was used to deposit a thin second layer (~20 μm) atop the wafer. UV treatment was performed by placing the foil mask featuring the contact lines and proper alignment of the layers. This step was followed by baking the photoresist, developing the unexposed areas, and silanization of the wafer with Chlorotrimethylsilane (Sigma Aldrich) to prepare the master mold for device fabrications.

Microfluidic chips were made by mixing PDMS elastomer base and curing agent (SYLGARD 184 Silicone Elastomer Kit, Dow Corning) in 1:10 ratio by weight. The mixture was fully degassed and then poured on top of the silicon master which was followed by another brief degassing step. The bubble-free mixture was placed inside a 70 °C oven for at least 3 hours. Later, the cured PDMS slab was peeled off the mold and cut into individual devices which were then punched for inlets and outlets. 1 mm biopsy puncher (Kai Medical) was used for all the connections unless otherwise was mentioned. Lastly, devices were bonded to microscope slides using air plasma for 50 sec followed by a brief baking at 80 °C.

PDMS wells were made using a blank silicon wafer. The resulting plain PDMS slab was punched concentrically with 6 and 12-mm punchers. The cut ring-shaped pieces were bonded to coverslips and used later for fluorescence imaging of the spheroids.

**MTB culture condition:** Wild type *Magnetospirillum magneticum* strain AMB-1 (ATCC 700264) and genetically modified AMB-1 to express GFP were used for this study. Bacteria were grown in revised magnetic spirillum growth medium (ATCC Medium 1653) containing 0.68 g of potassium phosphate, 0.37 g of succinic acid, 0.37 g of tartaric acid, 0.12 g of sodium nitrate, 0.035 g of ascorbic acid, 0.05 g of sodium acetate, 10 ml of Wolfe’s Vitamin Solution, 5 ml of Wolfe’s Mineral Solution, and 2-4 ml of 10 mM Ferric Quinate per liter of ultrapure water. pH of the media was adjusted to 6.75 by adding 1 M NaOH. Bacteria were passaged every 6-10 days by centrifuging the culture tubes at 9500 rpm for 10 min and resuspending in fresh medium. In order to provide microaerophilic condition, the optimal growth environment for this strain, 15 ml culture tubes were filled to the top leaving a small amount of headspace followed by sealing the screw cap with Parafilm before incubating at 30 °C.

Concentration of MTB samples was estimated through the established calibration curve correlating the optical density at 600 nm (OD₆₀₀) to the number density of the suspension. Pelleted bacteria were resuspended in PBS and transferred into standard polystyrene cuvettes. Optical density was measured using a spark multimode microplate reader (Tecan). To analyze
the responsiveness of the suspension to magnetic fields, same procedure was performed with a small NeFeB bar magnet on top once parallel with the light path (OD\|) and once perpendicular to it (OD\perp). The ratio of the measurements called $C_{\text{mag}} (OD\|/ OD\perp)$ indicates the extent of magnetic responsiveness which reflects the relative population of magnetic bacteria compared to non-magnetic ones.

**Collagen patterning in microfluidic devices:** Rat tail collagen type I (Corning, 354 249) was used to prepare the hydrogels for the transport studies. The stock solution was mixed with 10X PBS (ThermoFisher) and DI water to reach 2.5 mg/ml concentration. Following a brief vortex, 0.5 M NaOH solution was added in the end to neutralize the acidic mixture. Using 10X PBS with phenol red (made by dissolving 15.9 mg phenol red sodium salt (Sigma Aldrich) in 10 ml 10X PBS and sterile filtering the solution) provides a visual readout for adjusting the pH before injecting the collagen. Same procedure was followed for preparation of the fluorescently labelled hydrogels except that the final mixture contained 0.1 mg/ml of the TAMRA labelled collagen. The staining protocol is previously reported in []. All components were kept on ice during the entire process to prevent immature gelation of the collagen before filling microfluidic channels. To confine the collagen to the designated compartments in microfluidic chips using the contact line pinning strategy, only devices with restored hydrophobicity were employed for the collagen filling.

**MTB collagen penetration experiments:** Bonded devices with recovered hydrophobicity 2-3 days post plasma treatment were used for performing MTB penetration studies into hydrogels. TAMRA labelled collagen was added to the side chambers and incubated for 45 min at 37 °C. GFP expressing MTB were spun down and resuspended in PBS at the final concentration of $8.7 \times 10^9$ cells/ml. Central channels of the collagen-filled devices were injected with suspension of GFP MTB. Following 10 min of waiting time to let the device equilibrate, alternating RMF was applied for 30 min. Microscope images were taken at t=0, 15 min, and 30 min with respect to the magnetic actuation.

Images were analyzed in ImageJ to quantify penetration of the bacteria into the collagen. Rectangular regions of interest (ROIs) were defined using the signal from the collagen. GFP signal from MTB was then integrated throughout the collagen area at different time points and normalized by the respective t0 value. Average GFP signal as a function of the distance was also plotted to extract the penetration depth.
**Endothelial cell culture:** Human microvascular endothelium cells (HMEC-1, ATCC CRL-3243) were cultured in MCDB 131 medium (ThermoFischer) modified to contain 10 mM L-glutamine, 1 µg/mL hydrocortisone, 10 ng/mL EGF, 10% FBS, and 1% P/S. Cells were passaged 2-3 times a week at 70-80% confluency in T25 culture flasks. Each flask contained 4 ml of the growth medium and incubation was done at 37 °C and 5% CO₂. For each passaging, 1.5 ml of Trypsin-EDTA solution for 3-5 min was used to detach the cells from the substrate followed by adding 3 ml of culture medium to neutralize the enzymatic activity. Cells were then centrifuged at 320 x g for 3 min, and pellet was resuspended in fresh medium.

**Endothelialized channel fabrication:** Inner surface of the microfluidic chips were coated with Poly-D-Lysine (PDL) to enhance collagen attachment and confinement at desired locations and promote cell adhesion. All channels were filled with PDL solution at 1 mg/ml (Sigma Aldrich) and incubated at 37 °C for 4-8 hours. Next, devices were thoroughly washed with DI water and kept inside a 70 °C oven overnight to completely dry out. Coated devices rest at room temperature on the next day to restore the hydrophobicity.

Prior to the cell seeding, devices were UV-sterilized for at least 1 hour. To seed the cells inside the chips, corresponding chambers were first filled with the collagen gel. Devices were placed in a humidified petri-dish and incubated at 37 °C for 45 minutes. In the meantime, HMEC cells were collected from two confluent T25 flasks, counted using hemocytometer, and resuspended in fresh culture medium at 10⁷ cells/ml. Following gelation of the collagen, the cells were introduced into the central channels and incubated at 37 °C and 5% CO₂ for 2-3 hours for initial attachment to the surfaces. During this period, devices were tilted around 80 degrees alternately for half an hour on each side to promote cell adhesion at both collagen interfaces. Fresh culture media then was added into the channels and on top of all inlets/outlets. This step was done twice a day for three days until the monolayers formed on the surface of the collagen.

For immunofluorescence imaging, cells were washed by flushing the channels with PBS, followed by fixation in 4% paraformaldehyde for 15 min. 0.1% Triton X-100 (Sigma-Aldrich) was then used for 10 min to perforate the cells. Next, cells were incubated with 1% BSA in PBS at room temperature for an hour to minimize non-specific binding of antibodies. Channels were filled with VE-cadherin rabbit monoclonal primary antibody (2500S Cell Signaling Technology) diluted 1:500 in the blocking buffer and rest overnight at 4 °C. Following a proper wash PBS, Alexa488-conjugated anti-rabbit IgG (4412S Cell Signaling Technology) diluted
1:250 in the blocking buffer was introduced as the secondary antibody and devices were incubated at the room temperature for 1 hour. Cell nuclei were stained with Hoechst 33342 (10 mg/ml in water, Invitrogen) diluted 1:1000 in PBS. To stain the cell membrane, cells were incubated with 3,3’-dioctadecyloxacarbocyanine perchlorate (DiO, D4292-20 mg, Sigma-Fine Chemicals) at final concentration of 5 μg/ml.

**Computational model of endothelial translocation:** A numerical simulation in COMSOL Multiphysics was implemented to study the translocation of microstructures across an endothelial barrier under different conditions. MTB were modeled as rigid ellipsoids possessing a magnetic dipole moment using dimensions obtained from Multisizer data collected after *in vitro* translocation experiments. Endothelial cells were modeled as hyperelastic materials and stochastic opening of cell-cell contacts was incorporated to model dynamic gap formation that occurs in endothelial cell monolayers. The effect of the surrounding fluid was modelled through linear and rotational drag coefficients assuming Stokes flow regime. A time-varying external magnetic field was applied, and this was balanced with forces and torques arising from hydrodynamic and mechanical interactions with the viscous fluid and adjacent substrate.

In order to model the dynamic gaps forming stochastically between adjacent cells, a set of random parameters were generated for each simulation. These values determined the opening time of the junction and size of the gaps. Random gap size was within the range of 1.5 to 2.5 um (271) and the gap lifetime was set to 160 s (272). The overall simulation time was selected in a way that encompasses opening incidences of all gaps. Considering the size of the computational domain and translational motion of the bacteria, the simulation was carried out for 500 s.

**In vivo magnetic actuation experiments:** Female BALB/c nude mice (6-8 weeks, Charles River) were acclimatized for 3 weeks and inoculated subcutaneously in the hind flank with 5×10⁶ MCF-7 cells suspended in 8 mg/mL Matrigel at a volume of 100 μl. When tumor diameters exceeded 5 mm, mice were blindly randomized into various groups with a minimum of 3 mice per group. Tail-vein (intravenous) injections of MTB stained with a far-red proliferative dye were administered at a concentration of 1×10⁹ in 100 μl PBS. Mice were placed under anesthesia in the absence of magnetic actuation (control) or with tumors positioned in the workspace of the magnetic field generator and exposed to actuation at 20 mT and 14 Hz for 1
h (RMF). Mice were then returned to the cage for 24 h, after which the mice were euthanized and the tumor and major organs were harvested. For semiquantitative biodistribution analysis, far-red fluorescence signals were measured using an *ex vivo* fluorescence imaging system (Sapphire Biomolecular Imager, Azure Biosystems).

Harvested tumors were weighed and homogenized using a gentleMACS tissue dissociator (Miltenyi Biotec) (C-tubes) before being cultured for 8 days under the MTB culture conditions described previously. $C_{\text{mag}}$ measurements were performed to quantify the magnetic responsiveness of the samples. Optical density (OD) at 600 nm was measured with a magnet placed parallel (OD$_{\parallel}$) and then perpendicular (OD$_{\perp}$) to the light path. The $C_{\text{mag}}$ value was calculated as the fraction of OD$_{\parallel}$ and OD$_{\perp}$. 
4 Spatially selective magnetic actuation of living microrobots

The author declares that section 4.5 was conducted in direct collaboration with Tinotenda Gwisai. The author acknowledges the help of Daphne Asgeirsson for taking the SEM images. The author also acknowledges the contributions from Michael Christiansen in the design and construction of the magnetic setup.

4.1 Introduction

The ability to minimize off-target effects is a desirable feature for drug delivery platforms. This is often realized by either localizing the accumulation of active compounds to the target site or by selectively activating the portion that arrives in the targeted tissue. Driving forces underlying the desired selectivity can be internal or external. Internal forces utilize environmental cues in the tumor tissue to attract or activate the delivery system, whereas external energy sources like light, ultrasound, and magnetic fields can be leveraged in a manner that offers selectivity for the treatment strategy.

Although four different groups are created based on different combinations of localization strategy (selective accumulation vs selective activation) and the driving force (internal vs external), in practice these categories are not mutually exclusive. Combined methods may further enhance selectivity and open new avenues for taking advantage of synergistic effects between different approaches. For example, in the context of bacterial therapeutics, it is rational to ensure localized delivery since any off-target accumulation may result in complications associated with their toxicity. Simultaneously, due to their tumor tropism discussed in the first chapter, local amplification at the tumor site can act as a form of selective activation.

Owing to onboard sensing, bacteria are able to sense environmental cues and respond accordingly. In addition, synthetic biology has offered opportunities to genetically modify these bacterial robotic factories and fine-tune their tumor selectivity. However, targeted accumulation
of bacteria equipped with these functions is still contingent on the ability of their innate propulsive forces to overcome biological barriers posed by the tumor microenvironment. As a result, strategies for targeted introduction of external energy can potentially offer a much-needed element for enhanced selectivity of these living therapeutics.

### 4.2 Gating field as a means of localizing the actuation

Magnetic particle imaging (MPI) was introduced in 2005 as a tracer-based, high resolution imaging technique that relies fundamentally on the nonlinear response of superparamagnetic NPs (239). When exposed to an alternating magnetic field (AMF), NPs’ magnetization response shows an oscillating behavior containing the actuating frequency and higher harmonics (Figure 4-1A). Superimposing a sufficiently strong static field onto the AMF results in suppression of harmonics due to the saturation of the NPs (Figure 4-1B). A magnetic gradient field with a field free region (FFR), therefore, enables selective harmonic response only from the particles inside the FFR, thereby acting as a gating field or selection field. Magnetic moments of NPs outside this volume are pinned, preventing them from responding to the actuating field (275). Rapid movement of the generated zero point across the field of view is realized through three orthogonal drive field coils and a tomographic image is reconstructed by this spatial encoding (276).

Aside from diagnostic applications in cancer (277), the physical principle behind MPI has inspired several therapeutic applications driven by spatially selective magnetic actuation (242). Focused hyperthermia was introduced around a decade ago as a method to avoid hysteresis heating of off-target tissue, with an initial report achieving a 4.5 fold decrease in the specific absorption rate of particles placed a few centimeters apart within the actuating coil (278). Later theoretical studies on selection magnetic gradient fields present in MPI revealed possibility of millimeter scale resolution for the SAR peaks in space using current MPI technologies (279). This area of research was further advanced by the combination of MPI and magnetic hyperthermia in a single theranostic platform (280, 281). The capability of selectively actuating different vials in a therapeutic scan was also demonstrated. This integrated platform was also employed in an in vivo study for visualization and subsequently selective heating of a tumor in a xenograft mouse model (Figure 4-1C). Histological evaluation revealed lower thermal damage to the liver when the static gradient field was superimposed (240). More
recently, spatially controlled drug release from thermally responsive liposomes was studied using the same principle (Figure 4-1D) \((241)\). Through superposition of the gating field, it was shown that more than 40% release occurred inside the FFR while the off-target release was reduced to less than 10%.

These applications of spatially selected hysteresis heating rely on an AMF as the actuating field, similar to the original MPI detection principle. However, over the past few years, the feasibility of combining RMF with static selection fields has also been demonstrated in several studies \((282)\). At Phillips, one research group investigated the idea of spatially selective control of helical microrobots with the MPI coils \((283)\). Proof-of-concept experiments were performed using centimeter-scale objects such as screws which served as switchable source of radiation by moving up and down through a radiopaque shield. Offset static fields were used to control different screws individually. These experiments were followed by building a clinical scale setup capable of flexibly generating high fields and sufficiently strong gradients for selective actuation \((284)\). Guiding a magnetic drill through a pork tissue confirmed capability of the setup to generate large torques. Most recently, spatially selective rolling of NP clouds formed under application of RMF at the bottom of a tube was investigated inside a MPI scanner \((285)\). These studies demonstrate that scalable torque-based actuation can be rendered spatially selective when combined with the selection field. As a result, exposing MTB as efficient torque actuators to this actuation strategy could lead to a powerful targeted delivery scheme.
Figure 4-1: Biomedical applications of superimposing a magnetostatic gating field onto the time-varying actuating field. 

A,B) Physical principle behind magnetic particle imaging (MPI). Nonlinear magnetization response of superparamagnetic nanoparticles leads to suppression of the harmonics from the saturated particles located outside of the field free region (B) while the particles experiencing zero field respond with oscillating magnetization (A). 

C) Theranostic platform integrating MPI with the magnetic hyperthermia. Image guided localized heating of the tumor reduces damage to the off-target organs. 

D) Spatially controlled drug release through application of the selection field. Thermosensitive liposomes release the therapeutic cargo at FFR caused by heating of the NPs while the payload is retained outside of this volume. 

E) RMF generator of the clinical scale setup. F) Clinical scale setup integrating low-frequency RMF applicator and high-frequency drive coils for creating the imaging signal.
4.3 Design considerations in selection field scheme

Informed by the preliminary results from the predictive numerical simulations discussed in chapter 2, we sought to extend our understanding of off-target torque suppression by studying this phenomenon under different scenarios. In addition to the magnetic field direction with respect to axis of the channel, effects of the channel size and magnitude of the RMF were also assessed on silencing the rotational motion.

As expected, higher RMF magnitudes required a stronger magnetostatic field to reach the same level of suppression (Figure 4-2A and B). In terms of targeting resolution, this implies that higher accuracy, i.e. a smaller FFR, is more easily attainable as the magnitude of the actuating field is decreased under the same magnetostatic gating field. The general behavior of delivered torque density that drops off nonlinearly was replicated for both of the examined RMF values under various sizes of the channels. Average torque density in smaller channels exhibited a lower maximum and a less dramatic decay compared to millimeter size channels. These differences arise from the increased relative contribution of the wall effect in the torque density. Torque density becomes less uniform in small channels, which in turn needs a wider range of static fields to suppress all contributions across the channel width.

To consider the behavior of MTB under superposed fields, we also employed the previously established numerical model at the individual level for these living microrobots. This approach allowed a more detailed analysis of the angular displacement that a single bacterium as an independent microrobot undergoes (Figure 4-2C and D). By adding a relatively low magnitude of the magnetostatic component to the domain, the bacterium still underwent a full rotation, although the angular velocity became time dependent. At this stage, small rotations opposite to the direction of the rotating field were observed leading to back-and-forth motion of the dipole moment with higher portion of the forward rotation. As the contribution of the static field increased, it biased the rotation towards its own direction. This trend continued until the rotation was limited to a very confined oscillation around the magnetostatic field. These findings confirm the presence of similar behavior under the selection field for both swarm control and individual microrobots.

Examining the torque applied to an isolated microrobot under various magnitudes of the static field \((H_{st}=H_{RMF}\text{ and } H_{st}=H_{RMF}/4)\), comparable results were obtained for different RMF values (Figure 4-2E). At lower \(H_{st}\) values, the resultant oscillating torque featured flips with
lower periodicity than the actuating field and it was characterized with an average closer to the quasi-steady-state torque. When $H_{st}$ reached the $H_{RMF}$ value, oscillations around zero appeared to be the dynamic response of the microrobots to the superposed fields. Similar trends observed for various conditions prompted the idea of searching for a generalized curve applicable to all the conditions by nondimensionalizing the relevant variables (Figure 4-2F). Normalizing the torque density by the corresponding maximum at each condition and using the ratio of the magnetostatic field to the RMF for the x axis led to collapse of the curves into a single characteristic curve with intersecting points of 50% drop in torque density. This point represented gating field of $H_{RMF}/2$ which is determining factor in finding the resolution of selective actuation.

Lastly, to elucidate the role of the magnetosomes’ chain structure, we compared the above results with isolated magnetosomes (Figure 4-2G and H). With the same total volume fraction, isolated magnetosomes exhibited lower torque density due to the lost relatively large rigid dipole moment associated with the chains. Interestingly, the consistent 50% drop at $H_{RMF}/2$ were slightly disturbed here especially for smaller channels where other hydrodynamic effects compete with uniform alignment of the moments. Also, torque-density curves showed an undershoot in their decay towards zero.
Figure 4-2: Selection field requirements for suppression of the torque-driven transport. A, B) Decay of the average torque density across channels of varying widths as a function of differently oriented magnetostatic fields under RMF A) 12 mT and 14 Hz and B) 20 mT and 24 Hz. Angular displacement of individual microrobots exposed to different magnitudes of the static gating field with RMF C) 12 mT and 14 Hz and D) 20 mT and 24 Hz. E) Applied magnetic torque on a single microrobot under various actuating field and gating field values. Average torque drops to almost zero reflecting the transition from rotation with back-and-forth motion to symmetric oscillation. F) Nondimensionalized torque density curves of bacteria under different geometric and actuation conditions. Curves
nearly overlap leading to the presence of a generalized requirement for the definition of the FFR. G, H) Average torque density of isolated magnetosomes across channels of varying widths as a function of differently oriented magnetostatic fields under G) 12 mT RMF at 14 Hz and H) 20 mT RMF at 24 Hz.

4.4 Selective transport of NPs in vitro

As a first step toward empirically investigating selection fields in the context of rotational actuation of MTB, we performed a set of proof-of-principle experiments with an in vitro scheme to demonstrate selective actuation of bacteria under the selection field. Transport of NPs into a hydrogel was employed as the test case, with 200 nm NPs used as flow trackers indicating presence of convective transport resulting from actuation. For this purpose, a microfluidic chip consisting of five separate chambers arranged in a cross shape was designed (Figure 4-3A). Considering the 1 cm$^3$ working volume of the RMF generator and to ensure sufficiently uniform RMF exposure, the largest center to center distance of the wells was set to 1 cm with each well 4 mm in diameter. Circular contact lines were incorporated into the center of each well, enabling the confinement of collagen gels at the core and formation of a barrier-free interface with the surrounding liquid in the annular region. Four small NdFeB block magnets on the sides of the square were stably held by the PDMS and served as sources of the gating field. Using TAMRA-labelled collagen and green fluorescent NPs, successful compartmentalization was confirmed with confocal imaging (Figure 4-3A). SEM images of the master mold verified the presence of the microfabricated ridges in the center (Figure 4-3B and C). Similar to the previously shown microfluidic design in Chapter 3, these contact lines took up $\sim$10% of the total 150 μm height of the chambers.

To generate the desired field with a zero point in the center, magnets were configured to correspond to a sparse Halbach cylinder with $k=2$ (286). In the simplified version with only four magnets, arbitrarily defined north poles of the top and the bottom ones faced each other while the other two magnets were flipped with respect to the center and their south poles pointed inward. Finite element modeling of the magnetic field inside the working space using COMSOL Multiphysics enabled us to fine tune the generated magnetostatic field through changing the size and position of the permanent magnets (Figure 4-3D). The magnitude of the field gradient, anticipated magnetic forces between the block magnets, magnetic interaction with the coils generating the RMF, and resolution of the targeting zone were important factors that were considered in determining the final arrangement of the magnets (Figure 4-3E). While larger
magnets separated by longer distances ($d=22$ mm) provided a suitable size of FFR with lower field gradients, the smaller block magnets at a medium distance ($d=18$ mm) were picked for final experiments, since this configuration reduced interaction with the cores of the electromagnets at the expense of slightly higher gradients present in the working space.

Figure 4-3: Design and characterization of the in vitro platform to study selective transport of NPs. A) Schematic of the microfluidic design used for patterning collagen and NPs and exposing them to the selection field. Contact line pinning technique enables confinement of collagen (red) in the core of the chambers surrounded by suspension of NPs (green) in an annular form. B) SEM image of the microfluidic master mold from top. C) SEM image of the microfabricated ridge in a ring shape acting as the contact line. D) Computational modeling of the magnetic field generated by differently sized small block of NdFeB magnets. Size of the magnet influences the size of the zero point and magnitude of the gradients present in the working volume. E) Magnetic field profiles throughout the working volume for different sizes of the magnets positioned at varying distances. Small magnets at $d=18$ mm and large magnets at $d=22$ mm provide the desired resolution for selective actuation of target well.
Next, we used NP transport into the collagen as a measurable indicator of the extent of actuation scheme. Microfluidic devices embedding collagen gels in the center of the wells through application of the contact line pinning method were filled with MTB-NP suspension. The samples were exposed to multiple magnetic actuation regimes. Given the circularity of the collagen compartment and consequent lack of directional preference for the plane of RMF, a new actuation scheme called “sweeping RMF” was explored for this set of experiments. Under sweeping RMF, the plane of rotation went through one revolution during the actuation to cover all potential directions for the magnetically enhanced convection (Figure 4-4A). To better understand the performance of this actuation strategy compared to the previously applied constant RMF, a finite element-based model in COMSOL Multiphysics was established. Assuming porous material properties for the collagen, four different scenarios were compared with each other. In addition to the sweeping and constant RMF schemes, pure diffusion and sweeping RMF in the presence of external flow were studied (Figure 4-4B). The response of bacteria to the magnetic actuation was modelled as a torque-driven volume force acting on the fluidic compartment. Previously studied ferrohydrodynamics of the MTB suspension in chapter 2 provided the details of the flow profile and the volume force arising from the magnetic torque in such microfluidic chambers. Results indicated that the magnetically enhanced transport through application of the sweeping RMF can even exceed constant RMF (Figure 4-4B). This can be mainly attributed to the higher average concentration of NPs at the interface with collagen which was realized due to the better mixing of NPs under the sweeping scheme (Figure 4-4C). The integrated final concentration of NPs normalized by the concentration at the beginning of magnetic actuation, i.e. $t=15$ min, reflected the extent of magnetically enhanced transport (Figure 4-4D).

Informed by the results of the computational modeling, we proceeded with using sweeping RMF as the primary mode of actuation. The spatially selective scheme was then defined as superposition of the magnetostatic gating field on the sweeping RMF. Red fluorescent signal from the NPs was used to measure their concentration before and after the actuation (Figure 4-4E). Quantification of NPs present inside the collagen area showed significant increase of transport under magnetic actuation for both sweeping and constant RMF applied alone (Figure 4-4F). When a selection field was applied to the working space, NP transport into the off-target wells was reduced to the diffusion level, whereas more than 40% of the magnetically enhanced transport was maintained within the target region. An even higher
percentage is expected to be achieved in larger setups at animal or human scale, where a larger FFR should lead to lower gradients. In this initial experiment with a miniaturized setup, it important to recognize that independent effects of the magnetostatic field lead to a tradeoff between selectivity and overall suppression. Whereas the magnitude of the magnetostatic field serves to extinguish torque density, its associated gradients can also apply forces to MTB that draw them to the boundary and interfere with rotational actuation. Because the gradients associated with the zero point are on the order of 5 T/m in this setup, compared to 2 T/m for the mouse scale setup discussed in Section 4.10, this suppression effect is anticipated to be less physiologically relevant.
Figure 4-4: Magnetically enhanced selective transport of NPs into collagen gels. A) Schematic of sweeping RMF as an actuation scheme for isotropic targets. Plane of rotation undergoes one revolution during the experiment with sweeping RMF opposed to constant RMF where plane of rotation is fixed during the actuation period. B) Computationally resolved time evolution of NP concentration in collagen modelled as a porous material. Sweeping RMF slightly outperforms constant RMF, and presence of fluid flow inside the chambers has subtle effect on increase of the transport. C) Contours of NP concentration under constant and sweeping RMF. Sweeping velocity vectors lead to uniform increase of the transport through an effectively larger surface area while a separate band emerges inside the fluid at constant RMF indicating uniaxial mode of actuation. D) Simulation data of the fold change in NP concentration in the collagen compartment. E) Representative images of the NPs surrounding the collagen at the core of the chambers before and after the magnetic actuation (scale bar 500 µm). F) Fold change of signal from fluorescent NPs inside the collagen under different magnetic actuation schemes. Sweeping field shows better NP transport properties at the target compared to the constant RMF. Application of the selection field completely suppresses the off-target NP transport while it leads to a nonsignificant drop in the target area.
4.5 Targeted colonization of tumor spheroids

After observing initial indications of spatially selective actuation of bacteria by the selection field in collagen hydrogels, we proceeded by studying the spatially controlled bacterial accumulation in tumor spheroids as a more physiologically relevant model of cancer. Tumor spheroids are a type of widely used 3D models composed of densely packed cancer cells mimicking some of the features present in vivo, such as cell-cell interactions and gradients of oxygen and nutrients (287, 288). The microfluidic chip described in Section 4.4 was adapted for this study. 3-mm holes were punched only at the center of the wells, enabling placement of the spheroids inside the wells for the magnetic actuation and subsequent collection and incubation after the experiments (Figure 4-5A). Human breast cancer cells, MCF-7, were used to form spheroids of roughly 400 µm in diameter. Spheroids were then put inside the wells containing 25 µl of bacterial suspension prior to the experiment (Figure 4-5A).

Since spheroid colonization relies on penetration of bacteria into these 3D cancer models, we first verified spatial selectivity of actuation of MTB in response to the selection field by measuring their translational velocity inside the microfluidic chambers (Figure 4-5B). Because the translational velocity of MTB is torque driven, it can be used to directly assess their response as a function of position in the selection field. Results under 12 mT RMF and 14 Hz superimposed by the gating field from the smaller block magnets demonstrated the suppression of torque-driven motion of MTB when exposed to the selection field. The same experiment with larger block magnets exhibited generally the same trend in velocity magnitudes, with the stronger magnetostatic field improving the level of off target suppression but also reducing the extent of actuation within the target well (Figure 4-5B). This can be understood as different sizes of FFR which is defined with respect to the relative strength of the actuating field.

While the above results were obtained for bacteria in suspension where the main resistance force and torque arise primarily from viscous drags, the spheroid presents an additional barrier for successful penetration. As discussed in Chapter 3, optimal actuation frequency tends to shift in such situations in comparison to mere hydrodynamic coupling associated with the behavior of suspensions. This downward shift in optimal actuation frequency can be viewed as resulting from interaction with a medium of higher effective viscosity (Figure 4-5C). One strategy is to counteract this additional resistance with stronger rotating magnetic fields; however, this also increases the size of FFR given the $H_{RMF}/2$ rule of
thumb (grey dotted line vs blue dotted line). This reasoning is most applicable to actuation conditions close to the step-out frequency. Increasing the applied field magnitude in the absence of additional barriers would cause lower spatial confinement of the torque density, leading to a larger FFR and compromised resolution (red dotted line). With this argument, prepared samples were exposed to magnetic actuation for 1 hour where RMF was set to 20 mT and 14 Hz for these experiments. Small Neodymium block magnets were responsible for the gating field in all the selection field experiments. Here, motivated by the enhanced surface exploration hypothesis for translocation of bacteria across biological barriers, we again employed sweeping RMF as the main scheme of the actuation. Following magnetic actuation, the tumor spheroids were washed thoroughly in culture media and then incubated for 24 hours. Staining bacteria with far-red proliferative day allowed tracking of daughter cells within the course of the experiment.
Figure 4-5: Selective accumulation of the bacteria in the target spheroid. A) Schematics of the actuation setup and confocal image of the multiwell device. MCF-7 spheroids stained with Hoechst (blue) are immersed in wells containing suspension of far-red MTB. B) Translational velocity of bacteria exposed to different actuation schemes. Lower resolution of the zero-point generated by smaller magnets avoids drop in actuation at the target site while suppressing motion in off-target areas less completely. C) Simulated data of average torque densities in bacterial suspensions under various RMF parameters in low and high viscosity mediums. Increasing the field magnitude in the presence of higher resistance compensates for the loss in torque density under weaker fields and increases the required magnetostatic field to suppress off-target transport. D) Schematic of a spheroid and the focal planes used for confocal imaging and quantification of the bacterial accumulation. E) Accumulation of bacteria in tumor spheroids at different locations under various actuation schemes. Applying selection field results in significant but slightly higher than control bacterial accumulation in off-target spheroids. Target spheroid exhibits high accumulation of bacteria without any decrease compared to both sweeping and constant RMF.
Spheroids were transferred into PDMS wells on the day after for the fluorescence imaging. Z-stacks consisting of 10 µm distanced planes for the total height of 200 µm were taken to analyze accumulation of bacteria inside the spheroid (Figure 4-5D). It should be noted that light penetration and tissue opacity in confocal microscopy hinders collection of signals from the spheroid sections above this height. Quantification of far-red stained bacteria demonstrated significantly higher accumulation (2.3 fold) when exposed to the sweeping magnetic field compared to control (Figure 4-5E). The sweeping RMF again exhibited slightly superior, though nonsignificant, transport characteristics compared to the constant RMF. Most importantly, MTB colonization of the target spheroids did not experience any drop under application of the selection field, resulting in an approximately 3-fold increase compared to the control. However, at off-target sites, a significantly lower accumulation was observed in the presence of a superimposed gating field (Figure 4-5E). Clusters of bacteria inside the tumor spheroids were visible after 24 hours of incubation. Z-projected confocal images of the tumor spheroids confirmed selective accumulation of bacteria in the target spheroid by presence of such clusters (Figure 4-6).

Although the same trade-off discussed in the previous section between off target suppression and the introduction strong gradients was notable due to the scale of the system, the balance here was shifted towards maintaining full capacity of the magnetically assisted accumulation at the target region. This is mainly attributable to the change of effective FFR in these experiments. As a result, colonization of off-target spheroids was only partially suppressed (~1.5 fold change with respect to the control) compared to the NP transport studies in which off target wells behaved similarly to a negative control. These two observations together suggest that adjusting the size of FFR and choosing a balance between maximizing the delivery to the target and suppressing the off-target effects are important and adjustable aspects of implementing a selection field with rotational actuation.
Figure 4-6: Confocal microscopy images of the spheroids exposed to different actuation conditions. Spheroid in the center of the selection field exhibits clusters of far-red stained MTB 24 hr post actuation similar to all the spheroids exposed to the sweeping RMF. The rest of the spheroids at off-target locations in the selection field are sparsely colonized by the bacteria which occurs as the result of natural propulsion and field gradients. Control spheroids show slightly lower signal from the bacteria due to the absence of any magnetic forces and torques. (scale bar 100 µm).

4.6 Design of animal scale selection field setup

With the in vitro proof of concept experiments described in the previous two sections demonstrating the potential for using selection fields for targeted enhanced transport, we continued by designing a mouse scale setup suitable for in vivo studies. Not only did our findings suggest that performance should be improved for larger FFRs, but developing this setup is a crucial step towards developing a human scale system for potential clinical applications. Problems associated with scalability have hindered translation of many magnetically assisted strategies into clinics. Therefore, fabrication of an animal scale system helped to elucidate advantages and challenges that may emerge when scaling up the actuation strategy.

Informed by the preliminary results from the in vitro experiments, a conceptual design was first developed and studied. At this stage, we restricted our focus mainly to theoretical
considerations in order to determine the key requirements for such a system. Three main components were envisioned for successful targeted actuation in vivo each of which adds different functionality to the system.

**RMF Generator:** Spatially uniform magnetic field that rotates smoothly is desired for the proposed actuation scheme. Sufficient space for anesthetized mice exposed to magnetic actuation and easy access to them imposes another design constraint for this component. Generating rotating fields at larger scales has been a well-established practice for industrial applications such as in AC motors. Stators with three-phase windings are known to produce uniform RMF causing a magnetic torque on rotors. While different winding configurations result in various number of poles in the generated RMF and confer adjustability of the rotor speed, here we need two-pole RMF that generates spatially uniform RMF inside the stator and rotates with the same frequency as the input power. Such a coil can also outperform nested Helmholtz coils, which are another common RMF generators, in terms of uniformity of the field in the workspace. Also, the possibility of employing symmetrical windings in the stator structure ensures smooth rotation of a constant field without further adjustment of the input phase and power for each phase. As a result, a cylindrical coil fed by three-phase electric current (shifted by 120°) was chosen to generate the RMF while simultaneously providing a bed for the animal under anesthesia (Figure 4-7A).

**Gating field:** Arrays of permanent magnets were chosen again to be responsible for generating FFR with the desired resolution. Sufficiently scaled arrays magnets can be created by stacking blocks of NdFeB and Ferrite magnets, either separately or combined, to provide the required flux density for a given resolution. Considering a typical size of flank tumors employed for similar studies, 1 cm³ was set as the aimed resolution of the zero point. Since this resolution requires confinement of the zero point in 3D space, at least two Halbach cylinders placed at perpendicular planes were necessary to achieve this goal in both axial and radial directions of the cylindrical working space (Figure 4-7B). Again, configuration with k=2 was selected for each array, corresponding to the static field with isotropic zero point in the center.

**Displacement of FFR:** Previous in vitro studies were conducted in a way that the zero point was always located at the geometric center of the working space. Although experimental samples in other potential applications of this selective actuation scheme can be designed in a way that matches this constraint, such limitation negatively influences in vivo experiments with
animals where a stronger degree of variability in spatial positioning of the target is expected. Therefore, being able to displace the FFR offers a great benefit in terms of adaptability of the targeting strategy. This goal was met by supplementing the setup with a DC coil capable of generating uniform magnetostatic fields inside the working volume. Such an offset shifts the location of the zero point in space and enables targeting different points in space.

Axial positioning: While the DC coil moves FFR in the radial direction on the midplane of the cylindrical working space, axial positioning of the tumor with respect to the zero point remains as the last degree of freedom. A linear track with a sliding bed mounted on it is anticipated to ensure axial positioning of the tumor at the right point. Thus, precise targeting will be achieved first by centering the tumor axially and then moving the FFR radially to the right coordinate.

These components together formed a multilayer setup in which the DC coil was sandwiched between the AC coil and the permanent magnets (Figure 4-7B). This specific arrangement was informed by two design considerations. First, heat dissipation is a crucial consideration in such systems. Lack of proper cooling imposes limitations on attainable field magnitudes by the RMF generator and ultimately can damage the coils leading to malfunctioning of the system. As a result, heat needs to be rejected by active cooling through water circulation inside the copper tubes forming the DC coil. Having this component as close as possible to the AC coil guarantees proper heat transfer for the system under operation. In addition, as discussed in the proof-of-principle experiments, placement of strong magnets relatively close the center is associated with higher undesired field gradients. Although being further apart in space might diminish the ability of magnets to provide the desired resolution, a higher proportion of stronger magnets in the final arrays can circumvent this issue.

Preliminary finite element simulations of the magnetic field in COMSOL Multiphysics indicated that this arrangement satisfied most of the defined criteria. The application of current to the DC coils was also shown to move the zero point in the cross-sectional plane (Figure 4-7C). Lower axial resolution was also attributed to the lack of symmetry in the XZ Halbach cylinder where magnets were removed from both ends of the AC coil to provide access for positioning of the mice.
Figure 4-7: Conceptual design of a mouse scale selection field setup for in vivo studies. A) A three-phase AC coil exposes anesthetized mouse to uniform RMF. Three phase current with 120 phase lags is fed into the coil to generate a rotating field B) Multilayer configuration envisioned for the selection field setup. DC coil positioned between the AC coil and array of magnets is responsible for adding offset to the magnetostatic field from the permanent magnets and moving the zero point. C) Computational modeling of the static field from the Halbach arrays and the DC coil. Running current through the DC coil leads to shift of the zero-point location.

4.7 Design and fabrication of the three-phase AC coil

We started fabrication of the AC coil by designing a hollow cylinder containing six separate slots on the outer surface for three windings. To achieve highly uniform two-pole RMF, it is crucial to have uniform distribution of the conductors around the stator with minimal gap between them. Narrow dividers were incorporated on the outer surface of the stator to satisfy this need and facilitate manual winding of the coil (Figure 4-8A). The inner diameter was set to 5.5 cm allowing a mouse to slide into the working space. The CAD file constructed in SolidWorks was 3D printed in two pieces and then assembled. The space occupied by the magnet wires is of utmost importance as any increased distance from the center leads to difficulties in achieving the desired resolution from the gating field and compromises the
attainable RMF magnitude. Although relatively thick magnet wires can provide lower resistance and consequently better thermal characteristics, thin 0.5 mm wires were utilized to adequately compact this component of the setup. In order to obtain ideal uniform RMF with three-phase current, it is crucial to have equally strong contributions from each coil which are phase shifted by 120°. While this goal can be achieved for three geometrically different coils through addition of resistive and inductive loads, identical windings possessing a three-fold symmetry are able to satisfy this requirement without any additional adjustments in the electric circuit. By alternating periodically between the phases during the winding process, we managed to build the AC coil comprised of three windings with less than 3% variation in resistance and inductance of the coils (Figure 4-8C).

To test the performance of the AC coil and measure the attainable magnetic fields, coils were fed with phase-shifted electric currents. In this circuit, sinusoidal input voltages coming from the waveform generators of two Digilent AD2 devices were amplified and then passed through the windings (Figure 4-8D). Magnetic field measurements with a Hall probe exhibited linear response as the function of the input voltage from the waveform generator (Figure 4-8E). Measurements were carried out at different input frequencies within the range of interest for actuation of MTB, i.e. tens of Hz. Results indicated that there is no drop in amplification of the signal at frequencies above 10 Hz, and field magnitude of 20 mT can be achieved under $V_{in} \approx 850$ mV regardless of the input frequency.

Another challenge in generating ideal RMF for relatively a long period driven by separate waveform generators is to ensure accurate phase shift between the input signals. Using a single cross trigger led to simultaneous start of the signals (Figure 4-8F). However, tiny errors caused by imperfect synchronization accumulated over time, eventually resulting in deviation of the phase lags. This issue was even more pronounced at higher frequencies with more cycles in a certain time period. Using periodic cross triggering between the devices addressed this problem.
Figure 4-8: Three phase AC coil for generating RMF at large scale. A) CAD geometry of the stator designed for the three-phase winding. Dedicated slots to each winding ensure compact structure of the AC coil. B) Wound AC coil around the 3D printed stator. The central portion of the coil exhibits highest compactness providing space for the DC coil. C) Electrical properties of individual windings forming the AC coil. Symmetric period windings scheme yields less than 3% variability in electrical properties. D) Electric circuit of the RMF generating component. Phase-shifted input signals from Digilent devices are amplified and sent to the windings of the coil. E) Magnitude of RMF generated by the AC coil. Amplification of the signal from the waveform generators is consistent in the desired range of frequencies and fulfills the 20 mT requirement for the magnitude. F) Cross-triggering strategy for synchronizing input from two waveform generators. When two waveforms are generated with a certain phase lag from two different devices, minute deviations in each cycle propagate over time and disrupt the phase difference particular at higher frequencies. Periodic triggering resets the phase lags leading to consistent generation of uniform RMF.
4.8 Design and fabrication of the coils for DC offset

Analogous to the AC coil, the DC coil was designed to consist of three geometrically phase shifted coils comprised of copper tubing. The resultant three independent field vectors enable arbitrary movement of the zero point across the plane. The first step in building the DC coil was to estimate the required number of tubings. This parameter was set based on the range of movement that is desired for the zero point. Assuming linear behavior for the magnetic field in the vicinity of the FFR and the aimed 1 cm³ resolution under RMF of 20 mT, one can estimate the gradient to be 2 T/m. As a result, moving the zero point for 1 cm in any direction approximately requires a static field of 20 mT in strength. Geometrically, the inner diameter of the DC coil is set by the external diameter of the AC coil, and its outer diameter is constrained by the array of magnets. 2D computational modeling of the field produced by running 60 Amps current through a bundle of copper tubing arranged in two opposite sectors of a ring revealed that ~0.5 mT is generated per each turn (Figure 4-9A). Calculating the minimum number of turns to be 40, tube guides containing 49 holes were designed aiming to maintain copper tubes straight and close to the AC coil (Figure 4-9B). The 2D assumption in the simulation in addition to the geometrical constraints would in practice lead to lower field magnitudes from one phase. The contributions from other phases were predicted to compensate for that drop. Keeping the water-cooled copper tubes in close proximity to the magnet wires and anticipated use of thermally conductive potting epoxy ensures adequate heat exchange between these two components. The holder consisted of 6 rows for tubing and was fabricated by 3D printing. Two sets of 6 holders provided two parallel rings guiding the tubing around the AC coil.

While the tubes are wired in series electrically, hydraulically, they act as parallel path for the circulating water. Here, hydraulic circuit needed to become closed by first collecting parallel flows from a single bundle in one manifold and then merging the flows coming from six manifolds on the axial ends of the coils. Design of the manifolds was conducted by taking two geometric constraints into account. First, creating electrical connections on the tube ends requires access to all of them which is facilitated by having fewer number of rows. Second, copper tubes were forced to follow the curve imposed by the windings of the AC coil. Due to the outward expansion of the magnet wires at the axial ends of the central RMF coil, manifolds featured larger radius of curvature which enabled accommodating more tubes in a single row. As a result, final manifolds were designed with 3 rows of collecting holes for tubes and
contained flow splitters inside to uniformly distribute the flow across the tubes (Figure 4-9B). All manifolds were also 3D printed and then assembled to the tubes on both ends. Similar to tube guides, two sets of manifolds were considered for 6 pieces of the DC coil on both ends (Figure 4-9C). Each pair of tube bundles placed on the opposite sides of the AC coil was connected independently with series of 4 mm$^2$ wires and formed one coil by closing the current loop. Tubes and the connecting wires for half of each coil are shown in Figure 4-9D. Tubes of a single bundle were electrically put in series by connecting their ends to the corresponding ends on the opposite bundle. This series arrangement is necessary to obtain higher number of turns and consequently larger field magnitudes.

Three current controlled power supplies were used to power each subcoil independently (Figure 4-9E). These supplies were connected in parallel with a diode to protect against reverse current flow due to the inductive reactance in the circuit. In addition, chokes were added to the circuit to suppress the ripple current in the DC coil caused by the mutual inductance with the AC component (Figure 4-9F). In order to choose suitable chokes, two smaller coils were at first used for testing. Inductors in series with a total inductance of 250 μH were incorporated in the test DC coil. Results indicated that proper suppression of ripple current may be compromised at higher DC currents due to the saturation of the cores in chokes. Thus, although a very high inductance would be desirable, the final choke design in practice is likely to require relatively large iron powder cores with lower permeability and relatively modest number of turns to avoid saturation.

For heat rejection and temperature control, cooling water is anticipated to be circulated through either an air-cooled heat exchanger or a temperature-controlled reservoir. Thermal potting epoxy will be used after the assembly of both coils, both to maintain the structural integrity of the system and improve heat transfer with the AC coils.
4.9 Magic sphere, an array of magnets to create a magnetic zero point

The idea of using perpendicular arrays of permanent magnets were discussed before where confinement of the zero point in axial direction was eventually determined to be suboptimal. This was mainly because of lacking an array in the third plane, i.e. YZ, and partial
asymmetry of the XZ array due to the elongated geometry of the coils. An idealized scenario for having a relatively isotropic zero point in 3D space can be explained by a concept for an array of permanent magnets known as a “magic sphere” (289). This idea represents the conversion of Halbach cylinders into spheres with high uniform field in the enclosed cavity (Figure 4-10A). Although this has been introduced for Halbach \( k=1 \), here we extended this concept to a \( k=2 \) equivalent sphere which should generate an ideal gating field in 3D space (Figure 4-10B).

A magic sphere with a uniform internal field is derived from revolving the cross section of the \( k=1 \) cylinder around the axis that is aligned with the produced magnetization direction \( (z\text{-axis in Figure 4-10A}) \). Applying the same transformation to the \( k=2 \) cylinder will result in the sphere with zero point in its cavity (Figure 4-10B). Although perfectly isotropic zero point requires identical arrays in all directions, magnets cannot follow the \( k=2 \) pattern in all three orthogonal planes of symmetry (Figure 4-10C). In other words, revolving a 2D Halbach array around an axis does not result in the same array in the plane perpendicular to the axis of revolution. This consequence is translated into presence of a directionality for the magic sphere.

To better understand this phenomenon, we employed COMSOL Multiphysics to computationally study the magnetostatic field generated by such a sphere (Figure 4-10D). Informed by the geometric constraints arising from the AC and DC coils, average diameter of this sphere was set to 11 cm. First, this hypothetical sphere with continuous spatial distribution of magnetization was converted into a discretized sphere with two layers each containing a finite number of block magnets. Such a compact structure with permanent magnets leads in practice to powerful magnetic interactions between the magnets trying to dismantle the assembled sphere and minimize the stored energy. To mitigate this risk, stacks of ferrite block magnets (20 mm×20 mm×3 mm) were considered as the main constituent of the sphere. Axisymmetric simulation of the full sphere comprised of these magnets clearly reflected the presence of directionality. The sphere provided better resolution in the axial direction compared to the radial direction since the array formed in the transverse plane of a mouse deviates from the ideal \( k=2 \) Halbach array. Nonetheless, this sphere is predicted to meet the 1 cm\(^3\) criterion for the resolution in 3D (Figure 4-10E). However, AC and DC coils as the inner components of the multilayer setup dictated removing a cylindrical section from the center of the sphere. The decision for direction of the coils with respect to the axis of the magic sphere was made with
the goal of achieving higher isotropy for the FFR. In other words, the choice to align the axes of the coils with the axis of the sphere served to bring the radial and axial resolutions into better agreement.

Partial spheres were numerically modelled by excluding magnets intersecting with the geometry of the inner coils. Simulation results confirmed a greater loss of axial resolution than radial resolution resulting from this modified geometry. In addition, the zero point was no longer predicted to be <1 cm in the radial direction after this modification. Forming stronger elements as poles in the midplane, i.e. extending length of the stacks in the transverse plane up to the radius of the coils, served to partially restore the lost resolution. Stronger NdFeB block magnets (20 mm × 20 mm × 3 mm) were incorporated into the inner poles in the midplane to further enhance the resolution. This effect was approximated with an average remanent flux density for the stack which was extracted from these two types of magnets. This enhancement in terms of magnetic properties yielded the same resolution as the full sphere. Since strong magnetic interactions and difficulties in handling were anticipated with the use of strong magnets in this configuration, the same strategy was used for the stacks on the outer layer to create a favorable attractive force between the two layers holding them in place.

Informed by the results of the computational modeling, we designed geometry of the magic sphere with designated slots for the stacks of the permanent magnets the in SolidWorks (Figure 4-10F). Final CAD design was 3D printer in separate pieces and then assembled into two hemispheres. Empty slots in both inner and outer surface of the hemispheres were populated with magnet stacks epoxied in place (Figure 4-10G). Partial filling of the inner slots was also carried out to guarantee the maximum size of the desired FFR, which can be further enhanced by temporarily adding more magnets and adjusting the resolution.
Figure 4-10: Magic sphere as an azimuthally rotated Halbach cylinder to generate the desired magnetostatic field. A) Magic sphere represents the transformation of Halbach cylinder $k=1$ into 3D for uniform magnetic fields. B) 3D transformation of the Halbach cylinder with $k=2$ to create a zero point in 3D space. C) Cross section of the sphere resulting from revolution of the $k=2$ array. D) Computational modeling of the magnetic field generated by the $k=2$ magic sphere formed by ferrite block magnets. Geometrical constraints from the DC coil leads to a partial sphere that compromises the resolution. Adding additional magnets to the middle row and supplementing them with stronger NdFeB magnets restores the lost confinement of the zero point. E) Simulated magnetic field profiles in axial and radial directions. Higher resolution in the axial direction arises from deviation of the array in cross sectional plane from ideal $k=2$ arrangement. Profiles confirm attaining the desired 1 cm resolution by creating enhanced poles in the middle row of the sphere. F) Designed geometry for the skeleton of the modified magic sphere. Indentations on both inner and outer surfaces are incorporated as slots for stacks of block magnets. G) Fabricated modified magic sphere with mounted magnets. Inner and outer layers of magnets are assembled in way that guarantees 2-fold symmetry of the zero point.
4.10 Characterization of the selection field components

The DC coil and the partial magic sphere together generate the desired gating field capable of localizing the FFR to different locations within the working volume. After the design and fabrication steps discussed in the previous sections, components comprising the DC coils and the magic sphere were assembled to perform preliminary measurements of the static fields.

Two hemispheres populated with magnets were securely held together using cable binders. To measure the selection field generated by the magnets, a cube consisting of 64 hall sensors arranged in four equidistant planes was employed. Centering the cube inside the sphere allowed the measurements in a 3D volume encompassing the FFR. As predicted by numerical simulations, measured data indicated a slightly better resolution in the axial direction (~0.8 cm) compared to the radial direction (~1.2 cm). Adding or removing magnets in the slots located in the middle row of the inner layer permits adjustment of the size of the FFR according to the needs of an in vivo experiment.

The DC component was also tested after assembling the three coil pairs. Connecting all the copper tubes of one coil pair in series enabled single phase measurement of the generated field. The magnetic field measured by a Hall probe placed at the center of the coils showed that 7 mT can be achieved at minimum with a single phase. This value is anticipated to be slightly higher (10-20%) in the final setup where more compact assembly is required. Taking into account the contributions from the other coils, the attainable static field from the DC compartment is estimated to be approximately 15 mT. Examining the distribution of the magnetic field around the FFR reveals that the desired 2-cm range of motion can be fulfilled with combination of the fabricated DC coil and partial magic sphere.
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Figure 4-11: Characterization of the magnetostatic field generators. A) The assembled magic sphere for the magnetic field measurement. A cube of Hall sensors located at the center of the sphere measures the selection field from the magnets in a 3D volume. B) Measured magnetostatic field in a volume containing the FFR. Higher axial resolution is observed compared to the radial direction. C) The assembled DC coil comprised of three pair coils. One pair coil is electrically connected (red wires) for single phase measurements. D) Measured static field generated by one coil pair. At least 7 mT can be generated by each phase of the DC coil.

4.11 Conclusion

This chapter described the investigation of a concept for effectuating spatially targeted transport through the superposition of a magnetostatic gating field and an RMF. The approach ensures spatially selective application of magnetic torques to MTB by pinning their magnetic moments outside an FFR. Since combining an RMF and selection field for controlling swarms of independent actuators has not yet been well studied, we started with computational modeling of the torque suppression in off-target areas to better understand its requirements and limitations. It was determined that $H_{st} = H_{RMF}/2$ can serve as a general rule for sufficient drop of
the magnetic torque density, and is most applicable to actuation conditions close to the step-out frequency.

Using NP transport into collagen hydrogels as an indicator of the influence of actuation, we showed that spatially selective control is feasible at small scales. This finding was further supported by the subsequent study of selective bacterial transport into tumor spheroids as a more physiologically relevant in vitro model. Because these experiments required a zero point with linear dimensions of a few millimeters, higher gradient partially counteracted transport effects in the target area. Unlike many magnetic effects that are more readily exploited in miniaturized systems, the performance of this selection field strategy is expected to improve as the FFR is enlarged.

To demonstrate scalability of this approach, a mouse scale setup was designed for future in vivo studies, composed of three separate components that together form a multilayer system. The AC coil as the innermost component was shown to generate rotating fields above 20 mT. A partial magic sphere, consisting of an array of oriented magnets that creates the selection field, formed the outside layer of the system. Field measurements demonstrated the ability of this sphere to generate a FFR with the desired 1 cm$^3$ resolution. The DC coil positioned in the middle layer of the setup was comprised of three coil pairs responsible for adding a DC offset to move the FFR within the transverse plane of animal test subjects. Preliminary measurements indicated that static fields of up to 15 mT can be anticipated when all three phases are operating together.

While further assembly and characterization is needed before this prototype is ready for in vivo studies, the presented results supported the feasibility of scaled-up actuation and demonstrated promising features. Using efficient torque-driven microrobots under selection fields at human scales could pave the way for development of practical magnetically assisted delivery strategies in clinical applications.

4.12 Materials and Methods

*Selective NP transport experiments:* Following a minimum 2–3-day gap after the plasma bonding, microfluidic devices featuring five separate wells were used for the in-vitro selection field experiments. A slot to hold a magnet was also designed on each side for accurate positioning of the small magnets in selection field experiments. To generate a desired gating
field with zero point in the center, a simplified version of the Halbach cylinder $k=2$ was adopted using NdFeB block magnets ($5 \times 2.5 \times 1.5$ mm or $5 \times 2.5 \times 1.5$ mm, Supermagnete) (286). At the top and the bottom, arbitrarily defined north poles faced each other while north poles pointed outward on the left and the right sides. Initially the central part of each well distinguished by a ring-shaped contact line was filled with collagen. Following 45 min incubation at $37^\circ$C, they were stored in humidified petri-dishes at room temperature before start of the experiment. MTB were spun down and resuspended in PBS at the final concentration of $8.7 \times 10^9$ cells/ml. To quantify the NP transport, $1 \mu$l of red fluorescent NPs (FluoSpheres carboxylated microspheres, 0.2 mm) were added per 100 $\mu$l of the MTB suspension. Bacteria were then introduced into the annular part of the wells surrounding the collagen compartment. Prepared device underwent magnetic actuation for 1 hour while imaged at 0, 30 min, and 1 hour time points.

Image processing was performed in ImageJ (NIH), where the collagen area was determined with Analyze Particles command applied to the binarized image in the red channel. Fluorescent signal from NPs inside this ROI was integrated for different time points. The values of later time points were normalized by the time point 0 to compensate for any initial penetration of the NPs caused by the filling process. The ROI defined by the collagen was shrunk to form bands at certain intervals which enabled quantification of the signal as a function of the distance from the interface.

Magnetic actuation: An electromagnet setup consisting of 8 coils forming a hemisphere at one end as the working space was used for all magnetic actuations. The setup was integrated into an inverted microscope (Nikon Ti Eclipse), enabling live imaging of the samples under magnetic manipulation. The system can generate uniform arbitrary 3D magnetic fields within 1 cm$^3$ in space. Sweeping RMF was applied through application of the following input functions:

$$B_x = B \times \sin(2\pi f_2 t) \times \sin(2\pi f_1 t)$$
$$B_y = B \times \cos(2\pi f_2 t) \times \sin(2\pi f_1 t)$$
$$B_z = B \times \cos(2\pi f_1 t)$$

Where $f_1$ is the frequency of the out-of-plane RMF and $f_2$ represents the sweeping frequency of the plane of rotation. In order for the plane of rotation to go through one revolution during the actuation, $f_2$ was set to $1/3600$. Under the especial case of $f_2=0$, the actuation scheme
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turns into constant RMF with $yz$ as the plane of rotation. For experiments with the alternating RMF, one plane of rotation, i.e. $yz$, with switching axis of rotation between $x$ and -$x$ directions were used.

For application of the small-scale selection field, two sizes of NdFeB block magnets were investigated: $5 \times 2.5 \times 1.5$ mm or $5 \times 2.5 \times 1.0$ mm. Analysis of the MTB translational velocity was carried out under two gating fields generated by each of those arrays of magnets. The rest of the experiments were conducted only using the arrays consisting of the smaller NdFeB magnets.

**Selective tumor spheroid targeting experiments:** MCF-7 cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, ThermoFischer) supplemented with 10% FBS and 1% P/S. Ultralow adhesion plates (InSphero) were utilized to form tumor spheroids from MCF-7 cell line. Seeding density of 10,000 cells/well in 50 µL of growth media was used in 96 well plates. The well plates were centrifuged at 500 x g for 10 min followed by incubation at 37 °C with 5% CO$_2$. Size of the tumor spheroids reached ~400 µm after 3 days. DNA staining with Hoechst 33342 at a final concentration of 5 µg/mL in media was performed before the experiment for one hour at 37°C.

To stain the bacteria and be able to track subsequent generations, 2 µl of the far-red proliferative dye was added to 1 ml of bacteria suspension at $5\times10^8$ cells/ml. Following 20 min of agitation on a shaker while protected from light, the dye was deactivated using 100 µl of DMEM for 10 min. The bacteria were spun down and resuspended in 1 ml DMEM for the actuation experiments.

For these experiments, five-chamber devices were only punched at the center of each well with 3 mm punchers resulting in five wells for placing the spheroids. Corresponding slots for small magnets were cut and magnets were placed following the above-mentioned order for magnetization directions. Wells were rinsed with ethanol, air dried, and then exposed to UV for an hour for sterilization. Each well was filled with ~25 µl of MTB suspension, followed by release of spheroids at the top surface of liquid film formed by the suspension, which minimized any initial penetration of the bacteria. The spheroids settled at the bottom of the wells. Devices were then covered with a lid and mounted on the microscope holder to minimize contact with the surrounding air and avoid potential contaminations. Magnetic actuation at 20 mT and 14 Hz
was applied for 1 hour, after which the spheroids were collected from the wells, washed thoroughly with media, and then incubated in well plates at 37 °C and 5% CO₂ for 24 hours.

Spheroids were transferred into the PDMS rings after 24 hours and imaged with taking 200 μm high z-stacks in 10 μm intervals. To quantify the accumulation of bacteria in tumor spheroids, ROI was defined as the outer edge of the spheroid in binarized z-projected image in the DAPI channel. Far-red signal was then integrated throughout the resulting ROI at all planes to measure the fluorescence intensity which is correlated with the number of bacteria colonizing the spheroid.

*Computational modeling of magnetic actuation and NP transport:* COMSOL Multiphysics was utilized to model magnetic field distribution inside the working space and transport of NPs inside the microfluidic chamber. The Magnetic Fields No Currents physics interface enabled simulations of the magnetic field for the small-scale selection field setup. Four blocks representing the magnets were created according to their layout in the experiments while different values were assigned for size of the blocks and their mutual distances to allow for a parametric study. Each magnet was modelled as a permanent magnet possessing remnant flux density of 1.4 T and recoil permeability of 1.05.

To study NP transport, the geometry of a single well from the multi-chamber design was recreated. Governing equations from Stokes Flow and Transport of Diluted Species interfaces were solved for fluid velocity and concentration of the particles. The effect of magnetic actuation on MTB was modelled through application of a volume force corresponding to the torque-driven flow of bacteria in such chambers. Details are reported in Sections 2.7 and 2.8. Diffusion coefficient of the NP in liquid was calculated from the Stokes-Einstein relation ($D_0 = 2 \times 10^{-12}$ m²/s). Collagen gel was modelled as a porous material with porosity of 0.6. Hydraulic permeability was assumed to be $\kappa = 10^{-16}$ m² and diffusion coefficient was calculated by modifying the fluid diffusion coefficient according to the tortuosity model for effective diffusivity.
5 Conclusion and outlook

5.1 Summary of findings

This thesis has explored the use of MTB as a model system to investigate the potential of magnetically responsive living microrobots for enhanced convective delivery of nanodrugs or as direct therapeutic vectors for bacterial cancer therapy. Focus was set on establishing and analyzing methods for enhancing transport that can be applied in scaled up fashion, i.e. for deeply situated targets at human scale. This criterion is critical for clinical translation, yet neglected for many proposed magnetically based strategies, which often are limited to superficial targets. Computational and experimental models were created to study the benefits offered by local magnetic actuation of MTB through application of RMF. To elucidate the underlying principles of the transport mechanisms, MTB behavior under RMF was assessed at both the level of a swarm and at an individual level. A new magnetic actuation scheme was then proposed to selectively manipulate these bacteria at larger scales. Feasibility of this selective actuation strategy was confirmed by experiments conducted in a small-scale in vitro setup, and an animal scale prototype was designed and built for in vivo studies to follow. The following key findings resulted from these studies and are crucial for future work:

- MTB possess unique hydrodynamic and magnetic properties that make them ideal candidates as flow mediators. The chain structure of the magnetosomes leads to a magnetic order between the biomineralized iron nanocrystals, rendering the bacteria efficient torque-based actuators. Encapsulation of this chain in an elongated body not only avoids aggregation through magnetic interactions but also ensures efficient hydrodynamic energy transfer. These characteristics cause a suspension of MTB to generate two orders of magnitude higher flow per magnetic material compared to a synthetic ferrofluid.

- Unidirectional ferrohydrodynamic flow of dense MTB suspensions in microchannels can be remotely controlled via several well-defined operating parameters. A gradient of torque density across the channel is required to generate net flow in one direction. Such
a gradient can be achieved by applying a rotating gradient field or through gravitational settling of MTB.

- RMF applied to individual or low-density swarms of MTB in contact with boundaries induces translational motion and brings about enhanced surface exploration. When facing biological barriers with dynamic intercellular junctions like endothelial monolayers, higher surface exploration increases the likelihood of translocation through spontaneous gaps. The relationship between the timescale associated with the induced motion and the gap lifetime determine the success in converting surface exploration into effective crossing of the barrier.

- Saturation in magnetically enhanced transport arises when a relatively dense suspension of MTB is actuated with constant RMF. In addition to the number density, this saturation is influenced by the period of the actuation and the degree of settling inside the microchannel. To account for these effects, changing the plane of rotation during an experiment provides the opportunity to spatially control the direction and distribution of the enhanced transport, demonstrating the versatility of RMF-based schemes.

- Although it is challenging to replicate all physiological barriers in tissue models and this potentially causes a drop in the observed effects, few characteristics can play in favor of better performance in vivo. Presence of red blood cells may be interpreted as providing smaller conduits for higher effective volume fraction. Such physical constraint, despite shifting the optimal actuation frequency, leads to translational motion of a higher population of bacteria. This effect, among other factors, may help to account for the influence of magnetically enhanced transport that was observed in vivo.

- Selection gradient fields enable localization of actuation to confined areas. The target is defined as the region where the static field approaches zero. The magnetic moments of MTB outside this region are pinned and cannot follow the rotating field, causing suppression of the magnetic torque in off-target areas. Magnetic torque imparted by MTB drops off most rapidly by superimposing a static gradient field when they are actuated near their step-out frequency. This feature is translated into high spatial resolution of this selective actuation scheme.

- The versatility of selection field in adjusting the size of the FFR allows a balance to be struck between achieving enhanced transport at the target and reducing the off-target
effects. Size, position, and material of the static field generators act as the main parameters controlling the targeting resolution.

- Requirements associated with the size of the target at different scales as well as low magnitude of the RMF result in scalability of the actuation scheme for both animal and human studies. Furthermore, field gradients acting against the accumulation in the target site are diminished when the system is scaled up. This implies that not only is the use of a gating field for spatially selective actuation scalable, but it is expected to work more effectively for larger FFRs. The animal scale prototype developed and described in this thesis, featuring 1-cm resolution, is suitable for in vivo studies with mouse tumor models.

- Isotropic 3D confinement of the FFR is possible with special configuration of permanent magnets inspired by 3D extension of the Halbach cylinders. A dual-purpose DC coil enables movement of the FFR in the cross-sectional plane in addition to cooling the AC coil.

5.2 Future steps

This thesis has laid a foundation for studying enhanced drug transport using magnetically responsive living microrobots in combination with scalable actuation schemes. The future steps of this project will focus on the implementation and improvement of the individual elements of this drug delivery approach.

While bacterial cancer therapy benefits most from live, magnetic bacteria at early stages of the treatment, suppressed viability might be desired in the long-term for safety. As a result, other strains of bacteria that can more easily be modified to control the proliferation rate through genetic engineering could be considered. In this case, responsiveness to magnetic fields would be imparted to the bacteria through attachment or production of magnetic nanoparticles. In applications where self-propulsion and local amplification are not desired, inactivated bacteria or synthetic analogues would be most suitable. For example, in microfluidic pumping, if the goal is limited to the generation of consistent fluid flow over time, proliferation would lead to adverse time dependent properties. Another niche for this type of non-living microrobot could be the removal of microvascular obstructions. This intravascular application does not require tissue penetration and self-replication of the bacteria. However, if on-site production of a
therapeutic agent is desired, living bacteria possessing active gene expression machinery are necessary.

Regarding the spatially selective rotational actuation scheme, the first step will be to complete the assembly of the mouse scale selection field setup (Figure 5-1A and B). As previously described, a linear guide with a sliding block will be designed to adjust the position of the tumor with respect to the longitudinal axis of the setup (Figure 5-1C). Following the fabrication and characterization of the individual components of the prototype, accurate functioning of the assembled system will need to first be validated using in vitro models. A colorimetric or fluorometric assay could be designed where enhanced mixing through actuation of the bacteria would alter the spatial distribution or intensity of the readout in separate wells. For this assay, small volumes of bacterial suspensions mixed with an agent would be added to a 3D array of wells containing a viscous liquid. Quantification of the mixing, and thus the effect of the magnetic actuation, can be achieved through various means, such as pH dependent color change or spatial spread of a fluorescent marker. Comparing the mixing in different wells in the 3D array can provide useful information about the spatial selectivity in the working space of this prototype. Subsequently, in vivo experiments should be carried out by assigning different study groups to assess the effect of the selection field. Similar to the in vitro studies described in Sections 4.4 and 4.5, at least three groups (RMF, selection field, control) are required to examine the selectivity.
Another possibility for improving the proposed actuation regime involves incorporating feedback loops into the magnetic setup. As presented in this thesis, bacteria exhibit different dynamic behaviors at each stage of the in vivo journey. To optimize their performance for enhanced transport, live monitoring of MTB response to the external field would be highly beneficial. This measurement can be achieved by incorporating a small subsystem into the magnetic setup to enable inductive detection of MTB. The dynamic nature of RMF results in a time-varying magnetization from MTB which can be captured through an induced voltage. The main challenge in this process is anticipated to come from the low frequency nature of the response and absence of higher order harmonics. If effective, inductive feedback provides the opportunity to fine-tune the actuation parameters, leading to maximal drug transport following intravenous administration.
Numerous opportunities to further advance the microrobotic and instrumentation elements of the proposed drug delivery strategy can be anticipated. Contributions from other disciplines such as synthetic biology and medical imaging can potentially upgrade this strategy to a powerful multi-functional platform capable of addressing different requirements in an integrated way. By building upon the findings presented here on scalable, in vivo compatible magnetic actuation, progress towards translation of magnetically assisted living microrobots into the clinic could ultimately be facilitated.
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Appendices

A1. Effect of cobalt-doping on magnetic and hydrodynamic properties of MTB

This chapter has been adapted from the masters thesis of Alessandra Arizzi which was supervised by the author.

Unique magnetic properties of magnetosomes biomineralized by MTB opens many opportunities for their use in different biomedical and nanotechnology applications (117). Ideally, these magnetic properties can be further tailored for each specific application. Native magnetosomes contain magnetite nanocrystals with inverse spinel lattice structure where equal amounts of Fe$^{2+}$ and Fe$^{3+}$ ions occupy the octahedral sites and the Fe$^{3+}$ ions are located at the tetrahedral sites (290). Since the biomineralization process is well-regulated leading to a narrow size distribution and high purity of the crystals, researchers have tried to manipulate the magnetic properties in a controlled way by doping magnetosomes with transition metals like cobalt Co (291). This modification is partly motivated by non-metallophilic nature of the MTB due to high uptake of iron from the surrounding environment (292). Although contradicting results are reported about the effect of Co-doping on growth and size distribution of the magnetosomes, there is consensus that Co$^{2+}$ ions preferentially replace Fe$^{2+}$ ions in the lattice structure (293–296). This, in turn, increases the effective monocryalline anisotropy of the nanocrystals adding hardness to the magnetic properties by increasing the coercive field (296, 297). The cobalt content inside the doped magnetosomes was estimated to be in the range of 1% (298).

To investigate the altered magnetic properties and potential use of them as different types of torque actuators, we studied the effect of cobalt doping on the magnetic properties of strain AMB-1. Cobalt-doped bacteria were also cultured in the same growth medium, i.e. MSGM, except that cobalt-quinate (0.1 M) was also added to the media. Cobalt quinate was
made by dissolving 0.13 g of CoCl₂ in 100 mL of milliQ water, to which 0.19 g of quinic acid were added. In order to monitor the growth and responsiveness, OD₆₀₀ and Cₘₐ₉ values were collected at different time points during the culture. Also, VSM measurements were performed at the final time point, i.e. 10 days, which helped us estimate average magnetic moment of single bacterium at each condition using the following calculations:

\[
\text{OD}_{600,10 \text{ days}} \Rightarrow n_{\text{MTB,10 days}}
\]

\[
M_s = n_{\text{MTB}} \times m_{\text{MTB}}
\]

Different combinations of ferric quinate and cobalt quinate concentrations were tested. One general trend observed in the cultures was that cobalt doped samples exhibited stunt of the log phase and this behavior became more pronounced as the relative concentration of cobalt increased. Comparing OD and Cₘ₉ curves, it can be inferred that growth and responsiveness were not compromised in the samples with >10 μM cobalt quinate and total metal concentration of 20 μM.

![Figure A-1](image)

**Figure A-1**: Effect of cobalt doping on the growth and responsiveness of the bacteria. A) Growth curve for conditions with relatively low Co concentrations. Growth is not compromised by addition of the Co, and slightly improved under certain Co concentrations B) Growth curve for conditions with relatively high Co concentrations. Log phase is delayed and most of the conditions exhibit lower final MTB number densities. C) Cₘ₉ values, an indicator of magnetic responsiveness, over time for conditions with relatively low Co concentrations. D) Cₘ₉ time evolution for conditions with relatively high Co concentrations. Magnetic response is decayed as a result of high cobalt concentration.
This behavior was also reflected in the saturation magnetization and average magnetic moment of the samples with two (17.5 Fe/2.5 Co and 19 Fe/1 Co) even slightly outperforming the control. In addition, results of these two parameters indicated that relatively high cobalt concentrations not only negatively influence the growth, but also inhibit formation of magnetosome chains.

![Figure A-2: Magnetic properties of cobalt-doped MTB obtained by the VSM measurement under various cobalt concentrations. A) Saturation magnetization of the bacterial suspensions. Co-doped bacteria with low cobalt content outperform the control sample. B) Average magnetic moment of single bacterium. Low Co concentrations does not interfere with the formation of magnetosomes.](image)

Informed by these findings, we continued the next experiments with only two conditions showing the highest promise, i.e. 17.5 Fe/2.5 Co and 19 Fe/1 Co. To study the effect of Co-doping on the viability of bacteria, LIVE/DEAD BacLight Bacterial Viability Kit was used. Bacterial suspensions at OD$_{600}$=1.2 were prepared after 7 seven days of undisturbed culture. Imaging live and dead cell demonstrated similar viability among all conditions except the presence of slightly more dead bacteria for 17.5 Fe/2.5 Co which can be attributed to the higher cobalt concentration.
Figure A-3: Assessment of bacterial viability under doping with low cobalt concentrations. Dead bacteria are stained with PI (red). Both live and dead bacteria are stained with Syto 9 (green). Slight drop of viability is observed for 17.5 Fe/2.5 Co condition.

Knowing that the torque-driven fluid flow is mainly dependent on the magnetic moment and hydrodynamic size of the flow mediators, we also examined the size distribution of the Co-doped bacteria using Multisizer data. Although the general distribution resembled the one for control, interestingly, slight decrease in the mean size as the function of cobalt concentration was observed.
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Figure A-4: Effect of cobalt doping on hydrodynamic properties of MTB. Size distribution of bacteria from multisizer data for A) control B) 19 Fe/1 Co and C) 17.5 Fe/2.5 Co conditions. D) Mean diameter of bacterial population under different co-doping conditions. Slight decrease of the size is present as the function of Co concentration.

Lastly, motivated by the observed changes in the magnetic and hydrodynamic properties, though small, we sought to repeat the ferrohydrodynamic studies with the Co-doped bacteria. Results indicated that Co-doped bacteria were able to generate ~50% higher flows compared to the control close to the optimal actuation frequency (Figure A-5).

Figure A-5: Ferrohydrodynamic flow curves under different Co-doping conditions. Doped bacteria can outperform wildtype bacteria in generating torque-driven fluid flows.
A2. Enhanced cellular uptake and controlled release driven by MTB induced shear stresses

This chapter has been adapted from the masters theses of Thuy Trinh Nguyen and Alessandra Arizzi which were partially supervised by the author.

Living microrobots have the capacity integrate more than one functionality into a single platform. For instance, they can metabolically compete with tumor cells, modulate immune activity, and deliver therapeutic payloads at the same time. Torque-driven actuation of MTB serves as a potential source of shear forces that can be utilized to transiently increase permeability of the cell membranes, similar to what has been done with sonochemotherapy. Although higher shear stress is achieved with cavitating microbubbles under high-frequency ultrasound, developing an analogous strategy with magnetic fields removes the limitations associated with tissue absorption. Also, another form of sonochemotherapy is local release of drugs from microbubbles. Motivated by the capacity of RMF-induced MTB rotation in generating local shear forces, we investigated two scenarios: enhancing cellular uptake through the generated shear forces and inducing cargo release from shear sensitive carriers.

To study the enhanced cellular uptake, PDMS wells were coated with collagen solution (50 μg/ml) which was prepared by diluting 3.8 mg/mL collagen type 1, rat tail with 0.1% acetic acid. MDA-MB-231 cells were cultured in these wells for one day. For actuation experiments, wells were washed and subsequently filled with 200 μl of both the MTB suspension (OD 1.2) with propidium iodide (PI) at the concentration of 5 μg/ml. Bacteria were then exposed to in plane RMF at 94 Hz for half an hour to generate shear stress in the vicinity of the cell membranes. Since PI is normally taken up by dead cells due to the disrupted membrane, its uptake by live cells can be interpreted as transient pore formation in the cellular membrane. To potentially identify live cells with PI signal, 200 μL of Calcein was added to the suspension after the actuation.

Fluorescence imaging revealed that only a few cells were stained with both PI and Calcein. Interestingly, the control sample with bacteria and without any magnetic actuation also showed one cell with both signals. These results indicated that higher actuation frequencies are
required to generate shear forces relevant to the induced permeation. It is also possible that mechanisms other than rotational motion such as flagellar motion of the bacteria would contribute to the enhanced cellular uptake, a hypothesis that remains to be investigated.

Figure A-6: Cellular uptake of MDA-MB-231 cells in the presence of bacteria. A) Uptake of PI by live cells in the presence of bacteria actuated at 94 Hz. Only one cell shows uptake of both calcein AM and PI, a sign for pore formation in live cells. B) Uptake of PI by live cells in the presence of bacteria without magnetic actuation. One cell features overlapping fluorescent signals from PI and calcein AM.

The second strategy was to develop shear sensitive liposomes that can selectively release their cargo upon exposure to shear stress generated by the bacteria. NP platforms responsive to biochemical cues in the tumor microenvironment or external energies have been introduced. However, most of them still suffer from the diffusion-limited transport of the released cargo. With the capacity of bacteria in generating local convection, any locally released drug can also benefit from better transport characteristic on site. Shear responsive liposomes have been developed by few research groups where the application was more focused on stenosed arteries. Although average shear stress increases under such constrictions, the shear stress profile in tubes follows a linear variation under Poiseuille flow assumption. As a result, shear sensitive liposomes sense different levels of shear stress depending on their position inside the blood
vessel. However, bacteria can act as uniformly distributed sources of shear stress, potentially leading to a spatiotemporally-controlled release profile.

Liposomes formed by the self-assembly of mainly amphiphilic phospholipids serve as models for the plasma membrane. They have also been used as drug carriers due to their capability of encapsulating both hydrophobic and hydrophilic drugs. Several techniques have been suggested for making these vesicles sensitive to mechanical trigger. As an example, synthesizing lenticular-shaped vesicles potentially reportedly cause instabilities along their equator. Alternatively, mechanosensitivity of liposomes can also be accomplished with small membrane asymmetries present across the whole bilayer. One study used ternary lipid mixture POPC:POPG:cholesterol, which allowed structural changes under shear stress. Cholesterol was suggested to cause further structural heterogeneity, thereby increasing the sensitivity of the liposomes to shear stress. This formulation and the corresponding synthesis protocol from … was adapted as the basis of our studies.

POPC and POPG are unsaturated phospholipids with relatively large headgroups. Their low phase transition temperature, i.e. 2 °C, removes the need for heating the samples during the synthesis and contributes to their mechanosensitivity at room temperature. Briefly, calcein-loaded liposomes were made by using the lipid mixture POPC:POPG:cholesterol = 35.714 : 14.28 : 50 in molar ratio and hydration of the dry lipid film with 1.0 mL of 50 mM calcein solution in 50 mM Tris-HCl/100 mM NaCl buffer (pH 7.4) followed by stirring for 1h. After 8 freeze-thaw cycles the suspension was first extruded 11 times through a polycarbonate membrane with mean pore diameter of 400 nm and then 11 times more through a 200 nm one. Lastly, size exclusion chromatography was employed to remove nonencapsulated calcein molecules using a Sephadex G-25 M column with PBS.
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Figure A-7: Structure of the mechanosensitive liposomes. A) Molecular structure of the lipids forming the composition of the shear sensitive bilayer. B) Calcein loaded shear sensitive liposomes. Fluorescent signal of calcein is quenched at relatively high concentration inside the liposome. It fluoresce when diluted upon release from the liposomes.

Mean hydrodynamic diameter of 208 nm with a polydispersity index of 10.12% was measured for the liposomes using DLS. Elution profile indicated that the liposomes appear in the first few fractions and are distinctly separated from the non-encapsulated calcein. To perform calcein release assay, 0.2% and 1% Triton X-100 solutions were added to the liposomes as the positive control. As a detergent, Triton X-100 is known to lyse liposomal membranes and consequently trigger release of the encapsulated dye. Encapsulated calcein at the self-quenching concentration will dilute upon release, thereby increasing the fluorescent signal. Fractional release was calculated as follows:

\[
\text{Fractional Release} = \frac{I_t - I_0}{I_{\infty} - I_0}
\]

With \(I_0\) being the initial fluorescence intensity, \(I_{\infty}\) the maximal fluorescence intensity from triton-treated sample, and \(I_t\) the fluorescence intensity at timepoint \(t\). Successful

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encapsulation was confirmed through the release assay. Passive release assessment indicates that the new liposome formulation appears to be stable for a minimum of 5 days, reaching nearly identical fractional release values to the original liposome formulation of 10-15%.

![Graph A](image1.png)

**Figure A-8**: Characterization of the synthesized liposomes. A) Size distribution of the liposomes from the DLS measurement. B) Elution profile of the synthesized batch in the size exclusion chromatography. C) Calcein release assay. Treating with triton as a detergent leads to disruption of the membrane and release of the calcein. D) Passive release profile. Release fraction of below 20% is measured up to 5 days post synthesis.

To assess the mechanosensitivity of the synthesized liposomes, the samples were exposed to shear stress in capillary tubes under the flow controlled by syringe pump. Defining 2-5 Pa as the desired range of sensitivity, flow rates corresponding to three different wall shear stress values, i.e. 1 Pa, 4 Pa, and 10 Pa were tested. The test was also performed in two capillary tubes of different sizes to better characterize the liposomal release. Results indicated that the liposomes exhibit responsiveness to shear stress particularly for >1 Pa shear levels. This behavior though was more pronounced in the larger capillary tube. At this scale particles are
not uniformly distributed across the channel. Similar effects are observed in small blood vessels where white blood cells and platelets exhibits margination and red blood cells flow in the center. As a result, different distribution of the liposomes inside the two capillary tubes as well as nonuniform shear stress profile may be responsible for this discrepancy and need further exploration.

![Figure A-9: Calcein release from the liposomes exposed to the shear stress in capillary tubes. A) Release profile for three different wall shear stress values in a 864μm tube. Liposomes exhibit fairly linear cargo release in response to the applied shear stress. B) Release profile for three different wall shear stress values in a 510 um tube. Weaker response to the shear stress can be attributed to the nonuniform distribution of the stress and the liposomes across the channel.](image)

Next, we evaluated the potential of RMF-induced MTB rotation in applying shear stress on the liposomes leading to release of the payload. To this end, bacterial suspensions seven days after the culture were prepared at OD$_{600}$=1.2. Liposomes were then added to the suspensions at 1:100 ratio. Custom-made 4-mm PDMS wells were filled with 50 μl of the resulting MTP liposome suspension. The samples were exposed to RMF of 120 mT at 70 Hz for overall 10 minutes, and fluorescent signal were measured before and after the actuation as well as at two intermediate time points at 2 min and 5 min. Although overall increase over time was observed for the actuated samples, samples without the actuation, surprisingly, exhibited higher increase over time.
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Figure A-10: Casein release from the liposomes in a bacterial suspension. A) Release profile in the course of 10-min magnetic actuation. B) Release profile in the presence of bacteria without magnetic actuation.

To explore different potential contributors to the observed phenomenon, effects of being in contact with PDMS and bacterial motility were investigated. Interestingly, fixed bacteria triggered lower release compared to the live bacteria without any magnetic actuation. Comparing the fluorescent signals from the liposomes after 10 min of exposure to different conditions revealed potential role of flagellar activity in release of the payload. Detailed analysis of the shear stress levels generated by the flagellar motion and the extent of its contribution in the triggered release remains to be studied.

Figure A-11: Effect of flagellar motion on the release behavior of the liposomes. A) Release profile of the liposomes in the presence of fixed and live bacteria. Motility of the bacteria can be seen as a potential source of shear stress. B) Effect of PDMS and motility on the amount of released cargo. Highest release after 10 minutes is observed when the liposomes are added to PDMS wells with non-actuated bacteria.
A3. Nanoscale induction by single domain magnetic nanoparticles

This chapter is adapted from (299). The author acknowledges Michael Christiansen as the first author who wrote the manuscript. Finite element simulations are performed by the author.

Single domain magnetic nanoparticles are increasingly investigated as actuators of biological and chemical processes that respond to externally applied magnetic fields. Although their localized effects have often been attributed to nanoscale heating (300–303), recent experimental evidence suggests the need to consider alternative hypotheses. Here, using the stochastic Landau-Lifshitz-Gilbert (sLLG) equation and finite element modelling, we investigated and critically examine an alternative hypothesis that localized effects may instead involve the induced electric fields arising from the dynamical behavior of individual single domain magnetic particles.

First, we examine possible roles for induced electric fields in ferritin-based magnetogenetics. Despite its weak magnetic moment of about 300 µB, ferritin is predicted to produce surprisingly high electric field magnitudes in its immediate vicinity. Appreciable interaction with the gating charges of ion channel proteins can be ruled out, yet electric fields at the surface of ferritin’s mineral core may still offer a mechanistic clue linking Fe ion release and applied magnetic fields in magnetogenetics. In a second case, we consider the magnitude of electric fields occurring the surface of metallic nanoparticles that have been reported to enhance electrochemical production of hydrogen.

In order to model the induced electric field arising from the changing magnetic moment of nanoparticles, Maxwell’s equations were numerically solved using the finite element method for single domain magnetic moments evolving in time.

\[ \nabla \times \vec{H} = \vec{J} + \frac{\partial \vec{D}}{\partial t} \]  

A-1
\n
\[ \nabla \times \vec{E} = -\frac{\partial \vec{B}}{\partial t} \quad \text{(A-2)} \\
\nabla \cdot \vec{B} = 0 \quad \text{(A-3)} \\
\nabla \cdot \vec{D} = \rho \quad \text{(A-4)} 
\]

Here, \( \vec{J} \) represents the current density, \( \vec{D} \) is electric flux density, \( \vec{E} \) is the electric field, and \( \rho \) is electric charge density. Along with the following constitutive relations, these form the set of partial differential equations that describe distribution of electric and magnetic fields in the vicinity of the nanoparticles.

\[ \vec{D} = \epsilon_0 \epsilon_r \vec{E} \quad \text{(A-5)} \]
\[ \vec{B} = \mu_0 \mu_r \vec{H} \quad \text{(A-6)} \]
\[ \vec{J} = \sigma \vec{E} \quad \text{(A-7)} \]

\( \epsilon_0 \) is permittivity of vacuum, \( \epsilon_r \) is the relative permittivity, \( \mu_0 \) is the vacuum permeability, and \( \mu_r \) is the relative permeability. \( \sigma \) is the electrical conductivity.

For the magnetogenetics case study, a simplified 3D geometry of a nanoparticle located at the cytoplasmic side of the plasma membrane was reconstructed in COMSOL Multiphysics, where the Magnetic Fields interface was employed as the numerical solver. The computational domain consisted of four subdomains representing cytoplasm, nanoparticle, plasma membrane, and extracellular medium. Frequency-dependent conductivities and relative permittivities were assigned to each subdomain, as explained in (299).

Under the predicted dynamical magnetic moment of the nanoparticle, the set of coupled PDEs was solved for magnetic vector potential, from which the induced electric field was extracted using Faraday’s law. In instances where harmonic oscillation of the magnetic moment at fixed frequencies was considered, analysis was conducted in the frequency domain. A 100-ps time-dependent study was carried out by using fluctuation of the magnetic moment predicted by the sLLG model as the input to assess the transmembrane voltage as a function of time. Because the induced electric field is non-conservative, different paths were defined normal to
the membrane and adjacent to the nanoparticle to measure the induced voltage $V_{\text{ind}}$ between two points A and B using the following identity:

$$V_{\text{ind}} = -\int_A^B \frac{\partial A}{\partial t} \, d\vec{r},$$

where $\vec{A}$ represents magnetic vector potential.

Before applying Maxwell’s equations to results of the sLLG model available in (299), an upper bound for the inductive effects of ferritin in the vicinity of a neuronal membrane were considered under the assumption of harmonic oscillation at a characteristic frequency of 1 THz (Figure A0-12A). As a more general sweep of the relevant parameter space, induced electric fields were calculated at the surface of MNPs as a function of size (6 nm to 100 nm), magnetization (20 kA/m to 2000 kA/m), and frequency (1 GHz to 1 THz). Over the investigated frequency range, the peak electric field was found to scale linearly with the frequency, allowing the representation shown in (Figure A-12B), in which induced electric field strength is normalized to frequency.

Finally, using the model of the dynamic behavior of the moment of ferritin, we considered the voltage induced across the neuronal membrane at the location of the voltage gated ion channel (Figure A-12C). The voltage signal is found to be on the scale of 10-5 mV, far too small to supply a significant perturbation to the gating charge of the voltage gated ion channel, which would require 10s of mV (Figure A0-12D). The additive $dB/dt$ signal arising from multiple equidistant noninteracting ferritins increases with the square root of their number (299). This effectively rules out the possibility that the combined effect of neighboring ferritins could increase this value by many orders of magnitude. Moreover, even if the magnitude of the perturbative membrane voltage had been far larger, it is not clear whether its frequency in the 100s of GHz would have allowed it to influence the conformation of the voltage gated ion channel. Despite the appealing logical features of ferritin acting as source of inductive perturbation on the neuronal membrane for magnetogenetics, our analysis suggests that this hypothesis can be definitively ruled out.
Figure A-12: Potential role of ferritin in magnetogenetics. A) Induced electric fields are plotted for a ferritin moment near a membrane fluctuating at 1 THz. B) A generalized parameter space considers the magnitude of the electric field induced at the surface of hypothetical single domain particles spanning a size range from 6 to 100 nm diameter and a magnetization from 20 to 2000 kA/m. C) Schematic of a ferritin in the vicinity of a voltage gated ion channel. D) The transmembrane voltage predicted from electric fields induced by ferritin is shown for 100 ps of dynamical behavior predicted by the sLLG model. The magnitude of this transmembrane voltage, considered at the center of a neighboring voltage gated ion channel, is too weak to plausibly perturb the gating charge.

While ferritin may not act appreciably on the gating charges of nearby channel proteins, local induced electric fields may still be hypothesized to play some alternative indirect mechanistic role in magnetogenetic stimulation. Ferritin is primarily an iron storage protein and recent experiments suggest that the release of Fe^{2+} ions from its core and subsequent oxidation of lipids are likely involved in the indirect stimulation of TRPV channel proteins (Figure A-13A) (304, 305). A mechanism for magnetogenetics underpinned by wirelessly stimulated iron ion release would be more satisfactory if the roles of intrinsic magnetic behavior of ferritin and externally applied magnetic fields in this process could be elucidated. Thus, using our model of ferritin, we additionally considered induced electric fields in the direct vicinity of its mineral core.

One additional aspect to consider for the induced fields so close to the center of ferritin is the distribution of magnetization within its mineral core. The magnetic properties of ferritin
have variously been attributed to uncompensated surface spins or to the existence of internal magnetic phases (306, 307). To account for these possibilities, we considered the case of magnetization confined to the outermost layer ("magnetized shell") or to a small central region ("magnetized center") in addition to the assumption of uniform magnetization (Figure A-13B). Because precession had been found to dominate the inductive behavior of ferritin (299), the electric field was simulated for stable precession at the dominant frequency of 400 GHz. The magnitude of the expected induced electric fields converges at the surface of the particle for each of the three cases of magnetization distribution that were considered and similarly diminishes with distance (Figure A-13C).

![Figure A-13: Potential role of electric fields induced at the surface of ferritin in Fe ion release. A) Alternating magnetic fields is suggested to trigger the release of Fe²⁺ from ferritin, leading to local lipid oxidation of the membrane that indirectly stimulates the channel protein. B) Induced electric fields are considered for stable precession at 400 GHz assuming different distributions of magnetization within ferritin, including uniform distribution, uncompensated surface spins, or a more strongly magnetized inclusion. C) Variation of the magnitude of the peak induced electric field magnitude as a function of distance from the center of ferritin is plotted for the same three cases.]

Next, we used our computational model of the magnetization dynamics of single domain MNPs to consider whether enhanced nanoscale electric fields are induced by catalytic MNPs during magnetization reversal events modulated by an AMF. Because the timescale of reversal events is far shorter than the period of a typical AMF (100s of ps versus a period of 10^7 ps for a 100 kHz AMF), behavior observed at constant applied fields near the minimum magnitude
needed to trigger reversals should reflect behavior during reversals that occur during hysteresis. A representative example of the time evolution of magnetic moments during an isolated reversal event is shown in Figure A-14A, demonstrating a combination of precession and thermal fluctuation.

Considering purely precessional motion of the magnetic moment of the catalytic MNPs at 4.4 GHz reveals a crucial feature of nanoscale induction on the surface: dependence on the angle of precession (Figure A-14B). Inductive effects can be anticipated to depend substantially not only on the frequency, moment, and size of an MNP, but also on the instantaneous angle between its moment and the axis of precession. Angles that align the moment with the axis of precession, corresponding to $|\alpha_z| = 1$, suppress induced electric fields, whereas electric field magnitude is maximized when $\alpha_z = 0$ (Figure A-14B). In turn, this suggests that MNPs confining magnetic moments to angles near an easy axis between reversal events should experience a momentary increase in induced electric fields when undergoing precessional motion as they pass through low values of $|\alpha_z|$. Using our sLLG results for all of the reversal events observed at 20 mT in the $\kappa = 0.8$ case ($N = 14$), the induced electric fields were modelled on their surfaces for a duration of approximately 4.5 ns, centered on the reversal event. Because the timestep for the sLLG had been chosen to be relatively large to ensure that long timescales could be simulated for extracting reversal behavior, a 5 timestep averaging filter was applied (approximately 0.36 of a single period of precession) before simulating induced electric fields. Figure A-14C shows the mean value of the maximum electric field magnitude on the surface of the MNPs as a function of time, with this value clearly elevated immediately before and after the reversal event. Taken together, these results suggest that for MNPs capable of effectively thermally trapping moments, the magnitude and extent of induced electric fields are maximized during reversal events.
Figure A-14: Elevated induced electric fields on the surface of catalytic MNPs during reversal events correlated with hysteresis. A) An example of an extracted reversal event representing the kind expected during hysteresis heating is shown. B) Modelling the electric fields induced by stable precession of the magnetic moment at 4.4 GHz indicates considerable dependence on the angle of precession, shown by electric field plots for $\alpha_z = 0.0, 0.1, 0.4, 0.8$. Maximum electric field magnitude and surface averaged electric field magnitude (abscissa) are shown as a function of $\alpha_z$ (ordinate). C) Maximum electric field magnitude induced at the surface of the catalytic MNP versus time. The mean of all reversal events observed at 20 mT is shown ($N = 14$), with the empirically bootstrapped 95% confidence interval for the mean shaded. D) Time-averaged maximum induced electric field values were found for the reversal events represented in C) with averaging windows of 500 ps duration at the times ($-2.0$ ns, $-1.5$ ns) “before,” ($-0.25$ ns, 0.25 ns) “during,” and (1.5 ns, 2.0 ns) “after”.

A4. Local densification and boundary effects in the shear modulus measurements of hydrogels

This chapter has been adapted from (308). The author conducted the finite element modeling of the magnetic microrods in an elastic medium. The author acknowledges that Daphne Asgeirsson and Michael Christiansen wrote the main body of the paper.

As a method for microscale material characterization, rod-shaped magnetic micropores (μRods) entrapped in collagen hydrogels were exposed to a precisely controlled rotating magnetic field. After observing local variation in deflection of the magnetically actuated microrods in gels which is reflective of micromechanical heterogeneity, we tested whether this method can also distinguish variation in global stiffness values of collagen I hydrogels with increasing concentration (309). We applied our approach to determine effective shear moduli for concentrations of collagen ranging from 0.5 to 2.0 mg/mL (Figure A-15). For microscale analysis, samples incorporating μRods were subjected to an in-plane (XY) rotating magnetic field of 73 mT at 1 Hz. For each concentration, we analyzed three fields of view to extract effective shear moduli, summarized in Figure A-15a. Corresponding measurements of bulk shear moduli are shown in supplementary information of (308).

Overall, our data show a trend of shear moduli increasing with collagen concentration. The average microscale effective shear modulus for 0.5 mg/mL collagen was 0.8 kPa and increased consistently with concentration to 9.5 kPa at 2.0 mg/mL (Figure A-15a). By contrast, the shear moduli determined through bulk measurements ranged from 5.7 Pa at 0.5 mg/mL to 54.8 Pa at 2.0 mg/mL (supplementary information of (308)).

Previous literature indicates that the scale of measurement can partly explain this discrepancy, especially given the comparability of the dimensions of the μRods to the characteristic length scale of the collagen I networks studied here (310–312). Using our finite element model, we studied whether extrinsic factors could also contribute to the higher apparent rotational stiffness observed. By correlating fluorescence intensity with local concentration of TAMRA-labelled collagen, we studied the drop-off in intensity at the periphery of the μRods (Figure A-15b), finding that the collagen is approximately 15% denser at the surface of the
μRod. Increased collagen density is correlated with increasing stiffness. The finite element model was accordingly adapted to include elastic moduli that varied with distance from the surface of the μRods, dropping off at a characteristic length scale matching the one observed here. We found that the fitted rotational stiffness increased by a factor bounded by the fold increase in elastic modulus set at the surface of the μRod. For smaller increases in elastic modulus at the surface, as in the 2X case, this limit is approached more closely (Figure A-15c, right). In addition, we studied the influence of a nearby rigid wall on the deflection of μRods, finding that it contributes mainly to higher order stiffness terms and is most pronounced for μRods that are close to the wall and embedded in softer media (Figure A-15c, left). Both factors tend to systematically increase values determined for effective shear modulus. This reflects relevant aspects of the mechanical environment experienced directly by cells in a 3D ECM model (313). In fact, densification was even more pronounced for fibroblasts that had been cultured in identical collagen I hydrogels for 3 days, which exhibited an approximately 28% increase of collagen density at their surfaces (Figure A-15d).

Figure A-15: Embedded μRods probe local stiffness of a surrounding collagen matrix and are influenced by local densification and proximity of boundaries. (a) Effective shear moduli experienced by μRods in collagen matrices of varying concentration, based on observed deflection caused by a 1 Hz in-plane rotating magnetic field of 73 mT. Three fields of view were analyzed for each collagen concentration, ranging from 0.5 to 2.0 mg/mL. (b) Merged micrograph (i) of a 0.5 mg/mL TAMRA-labeled collagen matrix (confocal fluorescence) encapsulating a μRod (brightfield, false color). Scale bar: 10 μm. Inset shows an example of regions of integration used to quantify
fluorescence intensity as a function of distance from the μRod. Scale bar 1 μm. (ii) Fluorescence intensity (collagen fiber density) versus distance from μRod surface, normalized to intensity at 1.6 μm. (n = 19, shaded region represents s.d.). (c) Results from finite element model for a linear elastic matrix with a shear modulus of 500 Pa. Local densification and proximity to a rigid boundary are found to increase extracted effective shear moduli. (d) Merged confocal fluorescence micrograph (i) of a fibroblast cultured in 0.5 mg/mL collagen matrix for 3 days, stained for nucleus and actin cytoskeleton. Scale bar: 10 μm. Inset shows example of integration regions analogous to (b). Scale bar: 1 μm. (ii) Fluorescence intensity (collagen fiber density) versus distance from fibroblast surface, normalized to intensity at 1.6 μm (n = 17, shaded region represents s.d.).

Finite element modeling of the actuated microrods: A finite element (FE) model was established in COMSOL Multiphysics to simulate the motion of actuated microrods and numerically derive a function correlating effective shear modulus with the second order term of the rotational stiffness, accounting for geometric characteristics of the μRods. The correlation function is needed as analytical expressions proposed the relationship between rotational stiffness and shear modulus are limited to very small deflections.

The mechanical model was comprised of a rigid μRod surrounded by linear elastic material. The effect of geometric nonlinearities was included in the governing solid mechanics equations. A rigid dipole moment was assigned to the μRod aligned with its major axis. Magnetic torque exerted on the μRod was calculated as follows:

\[ T = mB\sin\theta \]  

Where \( m \) represents the magnetic moment of the μRod calculated based on the saturation magnetization of nickel and cobalt given the ratio obtained from EDX. B is the magnetic field which rotates at 1 Hz, and \( \theta \) indicates the phase lag between these two vectors.

Simulations were conducted for 2 s to ensure that the effect of inertia was negligible by comparing the first two cycles of actuation. To incorporate the effect of local densification, a mathematical expression for shear modulus was defined as a function of distance from surface of the μRod. The size of the densified area was extracted from fluorescent microscopy images and was set to 15 μm for the FE model. The influence of a nearby rigid boundary surface was also studied by positioning the μRod at different distances from a boundary while keeping the rest of boundaries far away from the μRod.

Resulting angular deflections of the μRod located in a material with bulk shear modulus \( G = 1 \) kPa at 100 mT under different conditions illustrate the contribution of both effects to the behaviour of the actuated μRod (Figure A-16).
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Figure A-16: Angular deflections of a μRod positioned at different heights relative to the bottom wall inside an elastic material with 1 kPa shear modulus. Displacements are larger in the absence of local stiffening A) compared to the rods experiencing 2-fold B) and 10-fold C) localized increase in the material stiffness at the surface.

Next, rotational stiffness of the material at different μRod lengths and shear moduli was extracted by using the 2nd order approximation of the equation for total energy of a microrod (308). This numerically derived matrix was used to estimate the effective shear modulus based on $k_2$ values obtained from the fitting algorithm for experimental datapoints (Figure A-17A). As a cross validation between the FE model and the numerical model based on the total energy of μRods, angular deflections from FE simulations were fed into the fitting algorithm (Figure A-17B). Comparing the estimated effective modulus with the value assumed for the elastic material in simulations reveals approximately 20% difference. This deviation was mainly attributed to the assumptions behind each modeling approach.

Figure A-17: Cross validation between two models. A) Matrix correlating shear modulus with rotational stiffness as a function of μRod length. B) Calculated shear moduli from the fitting algorithm fed with angular displacements from the FE simulations under prescribed bulk shear modulus.
A5. Establishing agar plate cultures of MTB

Culturing microorganisms on agar plates which are petri dishes containing gelled growth medium by adding agar is a common technique in microbiology. In this method, individual cultured microorganisms form distinct colonies where all daughter cells within a colony share the same genetic fingerprint with the originally plated cell. This feature enables counting microorganisms from liquid culture and also selecting for a specific genetic modification.

We sought to establish culture of MTB on agar plates in order to extract the number colony forming units (cfu) present in the harvested tumors from the mice. The final agar concentration for plates was set to 0.7% as initial trials with lower concentrations failed due to lack of proper gelation. To make the plates, highly concentrated (10X) mMSGM was first prepared separately using the same ingredients described in the main text except the mineral and vitamin solutions. 10X medium can be stored at the room temperature until final mixing with the water. 7 g of agar powder per liter of the final media was added to the distilled water followed by shaking the bottle to help dissolve the agar. It should be noted that due to water temperature at this stage, it is barely possible to achieve a perfect solution before the autoclaving process.

Agar solution was autoclaved which was followed by adding the 10X medium to the solution at a 1:10 volume ratio. While the obtained 1X solution was still at relatively high temperature, the solution was poured into the petri dishes. Each petri dish contained 84 ml agar mMSGM plus 840 μl vitamin supplement and 164 μl mineral solution which were added after pouring. Plates were left inside the biosafety hood until they fully solidified. They were stored at 4 °C in sealed bags before use.

In order to find out the optimal dilution required for growing distinct colonies, we started by plating approximately $10^1$, $10^2$, $10^3$, and $10^4$ bacteria per plate estimated from the OD measurements. The initial efforts of seeding the MTB on plates and incubating in hypoxia boxes were not successful. This was mainly attributed to the suboptimal oxygen concentration under those conditions. In general, flushing boxes or bags with nitrogen results in low concentrations of O$_2$ providing fairly anoxic environment, i.e. below 2%, for the culture. However, AMB-1 is a microaerophilic strain requiring 5-10% O$_2$ concentration as optimal growth condition.
Subsequent trials with using BD GasPak™ EZ Campy Container System Sachets placed with two plates in a single ziplog bag led to growth of sparse colonies after 6 days of culture. Given the fact that such sachets were designed for larger containers, we deduced the conclusion that although higher oxygen was present compared to the hypoxia box, the concentration did not reach the microaerophilic condition.

To obtain slightly higher O₂ concentration, next we used BD GasPak™ EZ Campy Pouch™ Systems comprising a small sachet and a resealable bag. Placing two petri dishes in a single bag and incubating at 30 °C culminated in overgrowth of bacteria after 5-6 days and lawn of bacteria were visible for different dilutions.

Figure A-18: Culture conditions for growing MTB on agar plates. A) Using ziplog bags with sachets designed for container systems leads to slightly lower than optimal oxygen concentrations. Relatively low number of colonies grow under this condition. B) BD GasPak™ EZ Campy Pouch™ Systems provide microaerophilic environment, the optimal culture condition for strain AMB-1. Overgrowth under this culture condition is reflected in the appearance of lawns of bacteria on the plates.
Curriculum Vitae

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- Last Semester GPA: 19.14/20 (4/4) *
- Overall GPA: 17.93/20 (3.88/4)

  Title of B.Sc. Thesis: Numerical Simulation and Test of Air-Cooled Battery Thermal Management System Using Thermo-Electric Coolers Designed for Hybrid Electric Vehicles (Score: 20/20)

High School Diploma in Physics and Mathematics/ Rouzbeh High School, Tehran, Iran

- Overall GPA: 19.3 (4/4)


Research Interests

Drug Delivery & Living Therapeutics

Cognitive Neuroscience & Brain Plasticity

Biofluid Dynamics & CFD Modeling

Cardiovascular Biomechanics & Medical Devices

Publications

Journal Papers


**Conference Proceedings**

• Mirkhani, N., Nguyen, T., Gwisai, T., Christiansen, M.G., Schuerle, S., “Ferrohydrodynamics of Bacterial Swarm Control”, MicroTAS 2019, Basel, Switzerland


• Mirkhani, N., Davoudi, M.R., Hanafizadeh, P., Javidi, D., Saffarian, N., "Combined Tilting Disc and Bileaflet Mechanical Valves Features, a Possible Solution for Better Hemodynamic Performance", Eurovalve Congress, Barcelona, Spain, January 2017*

• Masouminia, M. Mirkhani, N., Davoudi, M.R., Hanafizadeh, P., K. Sadeghy, "Effect of Aortic Mechanical Heart Valve Replacement on the Blood Flow Pattern in the Coronary Arteries and Valve Region", 2nd Conference on Novel Approaches of Biomedical Eng. in Cardiovascular Diseases, Tehran, Iran, February 2016


• **Mirkhani, N.**, Khosroshahi, S.S., Nakhaei, A., “Economic and Environmental Study of Solar Absorption Chillers in Domestic Applications”, the 2nd National Conference on Environmental Planning and Management EPM, Tehran, Iran, May 2012


* Best Presentation Award of the Conference

**Patents**


**Honors and Awards**

<table>
<thead>
<tr>
<th>Award Description</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awarded Direct Admission and Waived Tuition for Graduate Studies (M.Sc.) as a Top 10% Student in the Undergraduate Studies</td>
<td>2012</td>
</tr>
<tr>
<td>Member of “Iranian National Elites Foundation”</td>
<td>2012-Present</td>
</tr>
<tr>
<td><strong>Ranked 74</strong>th in the Nationwide Physics &amp; Mathematics University Entrance Exam</td>
<td>2009</td>
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<tr>
<td>among 320,000 Participants in Iran*</td>
<td></td>
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<tr>
<td><strong>Ranked 12</strong>th in the Nationwide Foreign Languages (English) University Entrance Exam</td>
<td>2009</td>
</tr>
<tr>
<td>among 280,000 Participants in Iran</td>
<td></td>
</tr>
<tr>
<td><strong>Full Scholarship</strong> to the University of Tehran for Undergraduate Program</td>
<td>2009-2013</td>
</tr>
</tbody>
</table>

*R Ranked 1st among the students who entered the Mech. Eng. Department

**Work and Research Experience**


• Establishing Collaborations with Clinicians Including a Team of Surgeons
• Numerical Simulation of Heart Valve Dynamics to Optimize the Orientation of the Leaflets
• Multi-phase Modeling of the Blood Flow in the Coronary Arteries in Case of Heart Valve Replacements

**Fluid Dynamics Engineer**, at Research & Development Department of Iran Industrial Pumps Co. (IIP Group) (2016-2017)

• Design and Simulation of Industrial Centrifugal Pumps for Oil, Gas, and Petrochemical Projects
• Modelling Double Suction Multi-Stage Pumps in order to Maximize Head of the Pump

**Research Assistant**, in the Hydraulic Machinery Research Institute (HMRI) at University of Tehran (2013-2014)

• Study of Ocean Wave Energy and Its Potential as a Renewable Energy Source
• Optimization of Wells Turbine Using Variable Chord length and Blade Solidity Using Numerical Simulation

**Research Assistant**, in the Vehicle, Fuel, and Environment Research Institute (VFERI) at University of Tehran (2011-2013)

• Design and Construction of a Battery Thermal Management System for the Hybrid Electric Bus
• Piping Design of a Cooling System for the Hybrid Electric Bus Produced by VFERI
Teaching Experience

**Practical Methods in Biofabrication**, Teaching Assistant, Health Sci. and Tech. Department  
2019-2021

**General Biomechanics**, Teaching Assistant, Mech. Eng. Department  
2014-2017

**Fluid Mechanics**, Teaching Assistant, Mech. Eng. Department  
2014-2017

**Incompressible Aerodynamics**, Teaching Assistant, Mech. Eng. Department  
2016-2017

Spring 2015

Spring 2013

**Solar Energy**, Teaching Assistant, Mech. Eng. Department  
Spring 2014

**Turbomachineries**, Teaching Assistant, Mech. Eng. Department  
Fall 2013

**Principles of Electrical Eng.**, Teaching Assistant, Mech. Eng. Department  
Fall 2013

2014-2016

Computer and Laboratory skills

Animal Studies
Tumor Innoculation & Health Monitoring / Inhalation Anaesthesia & Mice Dissection / Ex-vivo Organ Scan & Homogenization

Experimental Techniques
Photo & Soft Lithography / Immunostaining & Confocal Microscopy / Magnetic Manipulation / Cell and Bacteria Culture

Design and Modeling
COMSOL Multiphysics / ANSYS Fluent & System Coupling / Solidworks & FlowSimulation / CFX & CFD Post / Auto Cad

Image Processing
ImageJ / TurtleSeg / Mimics / MATLAB

Programming and Data Analysis
MATLAB / R / GraphPad Prism

Applied Software
Adobe Illustrator / VideoPad

Languages

**Persian**: Native  
**English**: Fluent (C1)  
**German**: Beginner (A2)

Extracurricular Activities

**Sports:**
- Playing Soccer: Championship in Two Intramural Competitions
- Swimming: Received Lifeguard Certificate from the Iran National Federation

**Reading:**
- Psychology Books
- Novels: Former Junior Member of the Children’s Book Council (CBC) of Iran

**Service:**
- Volunteer Teacher of Creativity for Elementary School Children (2010-2011)
- Invited Teacher of Physics in the City of Gorgan for Pre-University Students (2015)