Engineering escherichia coli for nanoparticle synthesis and targeting of colon cancer

Pasula, Rupali Reddy

2018

Pasula, R. R. (2018). Engineering escherichia coli for nanoparticle synthesis and targeting of colon cancer. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/75835>

<https://doi.org/10.32657/10356/75835>

Engineering *Escherichia coli* for Nanoparticle Synthesis and Targeting of Colon Cancer

Rupali Reddy Pasula

School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University in partial
fulfilment of the requirement for the degree of Doctor of Philosophy

2018

Acknowledgements

My sincerest gratitude lies with my advisor Professor Sierin Lim, who has been the primary source of constant support and encouragement through the highs and lows of research endeavors. Her time, vast repertoire of knowledge, and guidance have been instrumental in ensuring my success at every step of the journey. Prof. Sierin's contagious enthusiasm for research was motivational in keeping my spirits high and I am very thankful for the paradigmatic example she has set as a successful woman researcher and professor. She will always continue to inspire and motivate me throughout my career ahead.

I would like to thank my colleagues at BeANs lab, for maintaining a spirit of camaraderie, and working together to maintain a conducive and healthy environment for research. This journey would have been the same if not for all these wonderful folks! I take this opportunity to thank my post-doc Dr. Barindra Sana for suggestions and advice in my experiments. Dr. Ambrish Kumar's valuable suggestions provided at each step in designing, conducting and troubleshooting my experiments were highly insightful and informative.

In addition, I am grateful to Dr. Senthil Kumar from NUS, who has provided me with suggestions and immense help in carrying out my experiments. His guidance in magnetic characterization experiments has been invaluable and has helped me improve my understanding of the field immensely. I thank Prof Christian Nijhuis for his help during my candidature. My acknowledgements extend to Dr. Herng Tun Seng and Professor Jun Ding from department of Material Science Engineering in NUS for their immense help with magnetic characterization.

Special acknowledgements to Ambili, Vishnu and Mridul for valuable insights, long discussions and super fun lunches. Thanks for always being there for me, having an inquisitive and warm-hearted nature, and displaying a high level of professionalism and integrity. I thank Thinzar Win for getting me accustomed to the lab and helping me kick start my experiments here and for being an amazing friend and colleague.

In addition, I am extremely grateful to Prof. Susanna Leong for guiding me in the first year, when I comprehended the research methods and techniques. Also, it was during this period that I had the great opportunity to work with Dr. Jee Loon and Dr. Sun Zhoutong, who helped me inculcate various cloning techniques and design into my research.

My family has been my source of constant support and encouragement throughout this program. I would like to thank my parents, brother, friends and family members for instilling confidence, and motivating me to achieve success and dream big.

Table of Contents

Chapter 1: Introduction	1
Chapter 2: Literature Review	8
2.1 Part A: Biological Production of Nanoparticles.....	8
2.1.1 Microbial production of nanoparticles	10
2.1.1.1 Bacterial production	12
2.1.1.2 Production of nanoparticles by photosynthetic microorganisms ..	17
2.1.1.3 Production by yeast	19
2.1.1.4 Other scaffolds	20
2.1.2 Discussion and future prospects.....	21
2.2 Part B: Nanotechnology and Synthetic Biology in Therapeutic Applications	24
2.2.1 Nanoparticles in healthcare applications	24
2.2.2 Nanomedicine in cancer treatment.....	25
2.2.3 Magnetic resonance imaging	27
2.2.3.1 Nanoparticles in MRI	28
2.2.4 Synthetic biology	29
2.2.4.1 Synthetic biology in cancer treatment.....	30
Chapter 3: <i>In vivo</i> Iron Nanoparticle Synthesis in Engineered <i>E. coli</i>	32
3.1 Introduction	32
3.1.1 Chemical and biological synthesis of iron nanoparticles.....	32
3.1.1.1 Protein templates for nanoparticle synthesis	32
3.1.1.2 Iron metabolism in <i>E. coli</i>	34
3.1.1.3 Ferritin.....	36
3.1.1.4 Magnetite forming peptides	38
3.1.1.5 FeoB in <i>E. coli</i>	38
3.1.1.6 Ferrous iron efflux F (<i>fieF</i>)	40
3.1.2 Objectives of the chapter	41
3.2 Materials and Methods	43
3.2.1 <i>E. coli</i> strains and plasmids.....	43
3.2.2 <i>fieF</i> gene deletion.....	43
3.2.3 Plasmids in <i>E. coli</i> BL21(DE3)C+RIL and <i>E. coli</i> BL21(DE3)C+RIL/ Δ <i>fieF</i>	44
3.2.4 Bacterial culture	45

3.2.5	Cell lysis and protein purification.....	46
3.2.6	Protein and iron quantification.....	46
3.2.7	SDS-PAGE	47
3.2.8	Scanning electron microscopy	47
3.2.9	Magnetic measurements.....	48
3.3	Results and Discussions	50
3.3.1	<i>fieF</i> Knock-out Strain of <i>E. coli</i> BL21(DE3)C+RIL	50
3.3.2	Expression of <i>Archaeoglobus fulgidus</i> ferritin and <i>feoB</i>	51
3.3.2.1	SDS-PAGE analysis of gene expression.....	52
3.3.3	Total protein obtained from cultures.....	54
3.3.3.1	SDS-PAGE analysis of the semi-purified AfFtn protein samples	55
3.3.4	Iron concentrations from purified protein.....	56
3.3.5	Estimation of iron loading in AfFtn.....	57
3.3.6	Ferritin size measurements	58
3.3.7	Examination of the cellular morphology at different iron loading ...	60
3.3.8	Construction of magnetic <i>E. coli</i>	61
3.3.8.1	Magnetic characterization of aerobically and anaerobically grown <i>E. coli</i>	64
3.4	Conclusions	68
Chapter 4: Metal core Formation and Characterization of <i>Archaeoglobus fulgidus</i> Ferritin (AfFtnWT) and its Variant (AfFtnAA).....		69
4.1	Introduction	69
4.1.1	<i>Archaeoglobus fulgidus</i> ferritin and its variants	70
4.1.2	Nanoparticle synthesis in ferritin	71
4.1.3	Objectives of this chapter.....	72
4.2	Methods and Materials	73
4.2.1	Protein production.....	73
4.2.2	Cell lysis and purification	73
4.2.3	Protein estimation using Bradford assay.....	74
4.2.4	Iron loading in apo-AfFtnWT and AfFtnAA.....	74
4.2.5	Lyophilization of the purified protein.....	75
4.2.6	Dynamic light scattering	75
4.2.7	Transmission electron microscopy	75
4.2.8	Thermogravimetric analysis.....	76
4.2.9	Circular dichroism	76

4.2.10	Relaxivity measurements of AfFtnAA and AfFtnWT	77
4.2.11	Colorectal cancer cell proliferation.....	78
4.2.12	Prussian blue staining	78
4.3	Results and Discussions	80
4.3.1	Protein purification and iron loading	80
4.3.2	Protein cage size measurements.....	81
4.3.3	Thermal stability analysis of the protein cages.....	83
4.3.4	Circular dichroism of AfFtn.....	87
4.3.5	FTIR analysis of AfFtnWT and AfFtnAA.....	88
4.3.6	Magnetic characterization of AfFtnAA and AfFtnWT.....	92
4.3.7	Magnetic characterization of different loadings of AfFtnAA.....	93
4.3.8	Cytotoxic effects of AfFtnWT and AfFtnAA on colorectal cancer cells	98
4.4	Conclusions	104
Chapter 5: Ferritin Nanocages for Photodynamic Therapy		106
5.1	Introduction	106
5.1.1	Photodynamic therapy	106
5.1.1.1	Principles in photodynamic therapy	107
5.1.1.2	Photosensitizers	108
5.1.2	Ferritin nano-platform for molecular entrapment	113
5.1.3	Objectives of the chapter	114
5.2	Methods and Materials	116
5.2.1	AO loading in AfFtnAA and AfFtnWT.....	116
5.2.2	Photodynamic therapy – singlet oxygen generation and cell proliferation assay.....	117
5.2.3	Confocal imaging.....	117
5.3	Results and Discussions	119
5.3.1	Acridine orange loading in ferritin	119
5.3.2	Size characterization of AO loaded ferritin nanocages.....	120
5.3.3	Circular dichroism spectroscopy.....	120
5.3.4	Internalization of the ferritin nanocages	122
5.3.5	Effect of light activation on the AO loaded cages with colorectal cancer cells	124
5.3.6	Cell proliferation after light treatment	125
5.4	Conclusions	127

Chapter 6: Engineering <i>E. coli</i> to target and respond to colorectal cancer	129
6.1 Introduction	129
6.1.1 Bacterial two component signaling system.....	130
6.1.1.1 Histidine kinases in two-component signaling systems	130
6.1.2 EnvZ-OmpR two-component signaling system.....	133
6.1.3 VirA-VirG two-component signaling system	133
6.1.4 Role of synthetic Biology in engineering two-component signaling systems	134
6.1.5 Design considerations for two-component signaling systems	135
6.1.6 Invasin – Bacterial protein that binds to mammalian cells	135
6.1.7 Objectives of this chapter.....	136
6.2 Materials and Methods	138
6.2.1 <i>E. coli</i> strains and plasmids.....	138
6.2.2 Parts used in the plasmids	138
6.2.3 Plasmid construction	138
6.2.4 Scanning electron microscopy on co-cultured cells.....	140
6.2.5 Gentamicin protection assay	141
6.2.6 Confocal microscopy	142
6.3 Results and Discussions	144
6.3.1 Plasmid construction.....	144
6.3.2 Scanning electron microscopic analysis of bacterial adhesion to colorectal cancer cells.....	145
6.3.3 Internalization of engineered bacteria.....	149
6.3.4 Time course of bacterial entry into cancer cells through β 1-integrins...	152
6.4 Conclusions	159
Chapter 7: Concluding Remarks and Future Directions	160
7.1.1 Oral diagnostics for detection of colorectal cancer.....	160
7.1.2 AfFtn as a drug nano-carrier	161
7.1.3 Bacterial engineering to respond to colorectal cancer cells.....	163
References.....	165
Appendix I	188
Appendix II	190
Appendix III.....	191
Appendix IV.....	192

List of Figures

Figure 2.1: Milestones in nanoparticle research (above) (above) [34-44] compared with that of genetic engineering (below) [45-51].	11
Figure 2.2: Different modes of nanoparticle biosynthesis by microorganisms. a) CdS quantum dots produced in genetically engineered <i>E. coli</i> [52]. b) Gold nanoparticles produced on the membrane of <i>Synechocystis</i> sp. PCC 6803 [53]. c) Silver nanoparticles produced by <i>Pseudomonas aeruginosa</i> SM1 [54]. d) Silver nanoparticles on plasmid scaffolds [44]. e) Magnetite nanoparticles produced in <i>Rhodospirillum rubrum</i> [55].	12
Figure 2.3: A proposed model of biosynthesis of nano-Ag by periplasmic c-type cytochrome NapC in the silver-resistant <i>E. coli</i> strain 116AR [60].	14
Figure 2.4: Nanobiotechnology: A continuum of opportunity for nanotechnology in the life sciences (Reproduced with permission) [82].	24
Figure 3.1: Schematic representation highlighting the use of ferritin for nanophase material synthesis. a) native reaction of iron oxide cores yielding iron sulfide b) manganese oxide core formation in apoferritin c) ion-binding and hydrolytic polymerization leading to uranyl oxyhydroxide formation [154].	34
Figure 3.2: Surface representation of octahedral common ferritin protein (left) and tetrahedral AfFtn protein (right) (Reproduced with permission) [130].	37
Figure 3.3: Assembly of 24 subunits to form AfFtn protein (Reproduced with permission) [165].	38
Figure 3.4: Organization of the FeoABC operon in <i>E. coli</i> (Reproduced with permission) [171].	39
Figure 3.5: Predicted schematic representation of ferrous iron uptake by Feo of <i>E. coli</i> (Reproduced with permission) [171].	40
Figure 3.6: Structure of fieF gene product (PDB ID 2QFI).	41
Figure 3.7: Overexpression of AfFtn and FeoB along with the knock out of fieF in <i>E. coli</i> . Iron supplementation leads to formation of nanoparticles in the ferritin core.	42
Figure 3.8: PCR results to check for absence of fieF and kanamycin cassette. Lanes 1- 7 show knock out of fieF while BL21(DE3)C+RIL shows the presence of fieF.	50
Figure 3.9: Growth curve of <i>E. coli</i> BL21(DE3)C+RIL and <i>E. coli</i> BL21(DE3)C+RIL/ Δ fieF.	51

Figure 3.10: a) Growth curve of <i>E. coli</i> BL21(DE3)C+RIL with AfFtn and feoB, induced and non-induced, b) Growth curve of iron loaded <i>E. coli</i> BL21(DE3)C+RIL with AfFtn and feoB, induced and non-induced. Fe indicates 10 mM ferrous sulphate supplemented in the media.....	52
Figure 3.11: SDS-PAGE analysis of co-expression of AfFtn and FeoB in <i>E. coli</i> BL21(DE3)C+RIL (0.5 mM IPTG induction, 10 mM iron loading). P1 is the insoluble fraction, P2 is the membrane fraction and S is the final soluble fraction of the lysate.	53
Figure 3.12: Total protein concentration (ppm) obtained from cultures supplemented with a) 5 mM and b) 10 mM ferrous sulphate	55
Figure 3.13: SDS-PAGE analysis of the semi-purified lysates showing AfFtn (20 kDa) protein expression from a) DE3 host and b) Δ fieF host. (+ indicates ferrous iron supplementation in the medium)	55
Figure 3.14: Total iron concentration (ppm) obtained from cultures supplemented with a) 5 mM and b) 10 mM ferrous sulphate.	56
Figure 3.15: Iron per cage (24-mer) AfFtn when cells were supplemented with a) 5mM and b) 10mM ferrous sulphate.	58
Figure 3.16: DLS showing 15 nm iron loaded AfFtn in <i>E. coli</i> /AfFtn, <i>E. coli</i> /AfFtn/FeoB and Δ fieF/AfFtn/FeoB and control <i>E. coli</i> /AfFtn/FeoB.	60
Figure 3.17: High resolution Field Emission Scanning Electron Microscopy images of. a) <i>E. coli</i> /AfFtn/FeoB loaded with 10 mM iron, b) Δ fieF/AfFtn/FeoB loaded with 10 mM iron, c) <i>E. coli</i> /FeoB loaded with 10 mM iron, d) Δ fieF/FeoB loaded with 10 mM iron, e) <i>E. coli</i> BL21(DE3)C+RIL. Scale bar represents 1 μ m.	61
Figure 3.18: A 0.5 T permanent magnet was placed under the bacteria supplemented with 10 mM iron. The bacteria could be seen aligning with the magnetic field of the magnet placed under it.	63
Figure 3.19: Magnet placed under <i>E. coli</i> /AfFtn-m6A grown in aerobic conditions in M9 medium supplemented with 10 mM iron 4 hours post induction and grown overnight at 20 °C.	64
Figure 3.20: Induced magnetization hysteresis loop for iron core formed in a) aerobic conditions and b) anaerobic conditions at different temperatures. c) Magnetic hysteresis loop, saturation magnetization (Hc) and coercivity (Ms) of anaerobic iron supplemented sample obtained from the zoomed in image of (b). Green lines run through origin to show the symmetry about the axes. d) ZFC/FC curves for aerobic and anaerobic samples.	66
Figure 4.1: Crystal structure of a) open pore, wild type <i>Archaeoglobus fulgidus</i> ferritin and b) its closed pore mutant AfFtnAA.....	71

Figure 4.2: a) AfFtnWT (left) and b) AfFtnAA (right) size as measured by DLS for various iron loadings.....	82
Figure 4.3: Intactness of the cages as visualized by TEM with uranyl acetate negative staining. a) (Fe4800)AfFtnAA and b) ((Fe4800)AfFtnWT. Scale bar represents 50 nm.	82
Figure 4.4: TGA (solid) and DTG (dashed) plots of (a) apoAfFtnAA, (b) (Fe600)AfFtnAA, (c) (Fe1200)AfFtnAA, (d) (Fe2400)AfFtnAA, (e) (Fe3600)AfFtnAA and (f) (Fe4800)AfFtnAA.....	85
Figure 4.5: TGA (solid) and DTG (dashed) plots of (a) apoAfFtnWT, (b) (Fe600)AfFtnWT, (c) (Fe1200)AfFtnWT, (d) (Fe2400)AfFtnWT, (e) (Fe3600)AfFtnWT and (f) (Fe4800)AfFtnWT.....	86
Figure 4.6: Circular dichroism spectra of holo (different iron loaded) and apo- a) AfFtnAA and b) AfFtnWT.	88
Figure 4.7: FTIR spectra of apo- and holo-AfFtnWT with different iron loadings (above). Second derivative of the FTIR spectra of all iron loadings in AfFtnWT (below).	90
Figure 4.8: FTIR spectra of apo- and holo-AfFtnAA with different iron loadings (above). Second derivative of the FTIR spectra of all iron loadings in AfFtnAA (below).	91
Figure 4.9: a) longitudinal relaxation rate of (Fe4800)AfFtnAA, b) transverse relaxation rate constant of (Fe4800)AfFtnAA, c) longitudinal relaxation rate constant of (Fe4800)AfFtnWT and d) transverse relaxation rate constant of (Fe4800)AfFtnWT plotted against iron concentration. r_1 is the longitudinal relaxivity and r_2 is the transverse relaxivity.	92
Figure 4.10: Induced magnetization hysteresis loop of different iron loaded ferritin samples at 300K. d) Saturation magnetization (H_c) and coercivity (M_s) of different iron loaded samples.....	95
Figure 4.11: ZFC/FC curves for different iron loaded AfFtnAA. c) Temperature dependent magnetization in different iron loaded AfFtnAA. d) Hysteresis loop (zoomed in about the origin) of different iron loaded AfFtnAA.	97
Figure 4.12: Cell proliferation of a) HCT116 and b) SW480 (right) treated with varying concentrations of (Fe4800)AfFtnAA in the lower protein range of 0 to 0.1 μ M protein for 4, 8 and 24 hours. The viability of the control not treated with protein is 100% and the others are normalized relative to the control.	99
Figure 4.13: Cell proliferation of HCT116 and SW480 treated with varying concentrations of (Fe4800)AfFtn-AA (left) and (Fe4800)AfFtn-WT (right). a) HCT116 treated with (Fe4800)AfFtnAA, b) HCT116 treated with	

(Fe4800)AfFtnWT, c) SW480 treated with (Fe4800)AfFtnAA and d) SW480 treated with (Fe4800)AfFtnWT. Viability of untreated cells is set to 100% and other values are normalized against it.	100
Figure 4.14: HCT116 incubated with varied concentrations of (Fe4800)AfFtnAA for 24 hours stained with prussian blue.	102
Figure 4.15: HCT116 incubated with varied concentrations of (Fe4800)AfFtnWT for 24 hours stained with prussian blue.	102
Figure 4.16: SW480 incubated with varied concentrations of (Fe4800)AfFtnAA for 24 hours stained with prussian blue.	103
Figure 4.17: SW480 incubated with varied concentrations of (Fe4800)AfFtnWT for 24 hours stained with prussian blue.	103
Figure 5.1: Photophysical reactions in PDT [236].	108
Figure 5.2: Second generation photosensitizers [236].	110
Figure 5.3: Fluorescence image of the mouse osteosarcoma showing pulmonary metastatic lesions [283].	113
Figure 5.4: TEM image of a) (AO)AfFtnAA, b) (AO)AfFtnWT, c) (AO,Fe)AfFtnWT; and d) DLS plot of apo and AO loaded in both variants of AfFtn.	120
Figure 5.5: CD spectrum of AO loaded in both variants of AfFtn. Samples include unloaded apo-ferritin, AO loaded AfFtn, (AO,Fe) loaded AfFtn and iron loaded AfFtn	122
Figure 5.6: AfFtn-AA conjugated with FITC on N-terminus (green) incubated with HCT116 for 2 hours and stained for lysosomes (red) and nucleus (blue). a) Lysosomes red stain, b) green FITC-AfFtnAA, c) nucleus stained blue, d) merger of lysosomes and FITC-AfFtnAA and e) merger of lysosomes, FITC-AfFtnAA and nucleus.	123
Figure 5.7: Singlet oxygen generation measured with SOSG sensor in a) HCT116 (left) and b) SW480 (right). Samples include AO loaded, (AO,Fe) loaded and Fe loaded AfFtnWT/AfFtnAA. Controls without blue light treatment are represented by black bars and blue light treated are in blue bars.	124
Figure 5.8: Viability of HCT116 after 24 hours w/wo blue light treatment. Samples include HCT116 alone, iron loaded AfFtnWT and AfFtnAA, AO loaded AfFtnWT and AfFtnAA, iron and AO loaded AfFtnWT and AfFtnAA, and a positive control of free AO. Blue bars indicate blue light treatment and white bars are not treated.	125

Figure 5.9: Viability of SW480 after 24 hours w/wo blue light treatment. Samples include SW480 alone, iron loaded AfFtnWT and AfFtnAA, AO loaded AfFtnWT and AfFtnAA, iron and AO loaded AfFtnWT and AfFtnAA, and a positive control of free AO. Blue bars indicate blue light treatment and white bars are not treated.	126
Figure 6.1: The schematic of Tar-EnvZ chimeric protein, Taz showing EnvZ histidine kinase domain. TM is the transmembrane; DHp is a dimerization histidine phosphotransfer domain and CA is a catalytic ATP-binding domain.	132
Figure 6.2: InvVirA-VirG (above) and InvEnvZ-OmpR (below) engineered two-component circuits to bind to $\beta 1$ integrins. InvVirA and InvEnvZ are the histidine kinases, VirG and OmpR are the response regulators, and RFP is the signal output.	136
Figure 6.3: Engineered <i>E. coli</i> TOP10 expressing InvEnvZ and InvVirA at an MOI 50 incubated for 2 hours adhere to colorectal cancer cell line HCT116. Control experiment with non-engineered <i>E. coli</i> TOP10 cells show no binding. Scale bar for images on the left represents 10 μm and zoomed in version with scale bar representing 1 μm is presented on the right.	147
Figure 6.4: Engineered <i>E. coli</i> TOP10 strain expressing InvEnvZ and InvVirA at an MOI 50 incubated for 2 hours adhere to colorectal cancer cell line SW480. Control experiment with non-engineered <i>E. coli</i> TOP10 cells show no binding. Scale bar for images on the left represents 10 μm and zoomed in version with scale bar representing 1 μm is presented on the right.	148
Figure 6.5: Bacterial internalization study by gentamicin protection assay performed at different MOI ratios and co incubated with HCT116 and SW480 for 2 hours.	150
Figure 6.6: Percentage of bacterial internalization when bacteria at different MOIs are co-incubated with HCT116 and SW480 for 2 hours.	151
Figure 6.7: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with HCT116 for (a) 2 hours, (b) 4 hours, Nucleus is stained blue, integrins are stained green and bacteria is red.	155
Figure 6.8: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with HCT116 for (c) 6 hours, (d) 8 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.	156
Figure 6.9: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with SW480 for (e) 2 hours, (f) 4 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.	157
Figure 6.10: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with SW480 for (g) 6 hours, and (h) 8 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.	158

Figure 10.1: Hydrophobic interaction chromatograms for AfFtnWT with inset showing the SDS gel for cell lysate (L), supernatant after heat treatment (S), pellet after heat treatment (P), and fractions A4, 5 and A6. 20 kDa band corresponds to AfFtnWT monomers. 190

Figure 11.1: Standard absorbance plot of AO in 0 to 50 μ M range 191

List of Tables

Table 1: Cancer therapy nanomedicines in clinical stage [19]. (Reproduced with permission).....	26
Table 2: <i>E. coli</i> strains with respective plasmids and antibiotic resistance.....	45
Table 3: Iron per cage (24-mer) AfFtn obtained in the engineered bacterial strains.	58
Table 4: Estimation of the iron loaded per cage AfFtn-WT and AfFtn-AA.	81
Table 5: AO loading in AfFtn.	120

List of Abbreviations

ABC	ATP-binding cassette
AfFtn	<i>Archaeoglobus fulgidus</i> ferritin
AO	Acridine orange
ATP	Adenosine tri-phosphate
BB	Biobrick
BCM	Biologically controlled biomineralization
BIM	Biologically induced biomineralization
bp	Basepairs
CCMV	Cowpea chlorotic mottle virus
CDF	Cation diffusion facilitator
CHO	Chinese hamster ovary
CMFDA	5-Chloromethylfluorescein diacetate
CT	Cholera toxin
CTNNBI	Catenin beta I
DAPI	4',6-diamidino-2-phenylindole
DI	Deionized
DLS	Dynamic light scattering
DNA	Deoxy ribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPDP	Diphenyl isodecyl phosphite
DTG	Derivative thermogravimetric
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EPR	Enhanced permeability and retention
FC	Field cooling
FESEM	Field emission scanning electron microscope
FITC	Fluorescein isothiocyanate

FLP	Flippase
FRT	Flippase recognition target
FTIR	Fourier transform infrared spectroscopy
Fur	Ferric uptake regulator
GDP	Guanosine diphosphate
GI	Gastrointestinal
GTP	Guanosine triphosphate
GM-CSF	Granulocyte macrophage colony stimulating factor
HEK	Human embryonic kidney
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIC	Hydrophobic interaction chromatography
HMDS	Hexamethyl disilazane
HpD	Hematoporphyrin derivative
ICP-OES	Inductively coupled plasma optical emission spectroscopy
iPSCs	Induced pluripotent stem cells
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IR	Infrared
kb	Kilo basepairs
kDa	Kilo dalton
kV	Kilo volts
LB	Lysogeny broth
LSM	Laser scanning microscope
MB	Methylene blue
MOI	Multiplicity of index
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MWCO	Molecular weight cut off

OD	Optical density
Omp	Outer membrane protein
PAMAM	Polyamidoamine
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC	Phytochelatin
PCR	Polymerase chain reaction
PDB	Protein data bank
PDT	Photo dynamic therapy
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PNK	Phosphonucleotide kinase
ppm	Parts per million
PS	Photosensitizer
PVA	Polyvinyl alcohol
RBS	Ribosome binding site
RFC	Request for comment
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
shRNA	Short hairpin ribonucleic acid
SO	Singlet oxygen
SOSG	Singlet oxygen sensor green

SQUID	Superconducting quantum interference device
TAE	Tris-acetate EDTA
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
UV	Ultraviolet
ZFC	Zero Field Cooling

Summary

The past decade and a half stands witness to a remarkable growth in the techniques of forward genetic engineering in living organisms, thereby engendering synthetic biology as a major player in disease diagnosis and treatment among other biomedical applications such as vaccine development and microbiome engineering. There is a growing interest to combine both nanotechnology and synthetic biology in cancer treatment due the appealing features of drug delivery and diagnosis offered by miniature programmable robots.

Synthetic biology has been rapidly producing tools used in biomedicine by leveraging the various genetic tools at its disposal. This strategy has been extended to nanotechnology leading to the design of novel organisms capable of producing nanoscale materials with high precision. Microbes have been engineered to produce various natural and unnatural nanoparticles by borrowing the various available synthetic biology tools. Here, we report the first attempt to employ a synthetic biology approach to engineer the bacterium *Escherichia coli* to increase the iron loading in *Archaeoglobus fulgidus* Ferritin (AfFtn) by co-expressing an iron influx protein (FeoB) and knocking out an iron efflux protein (*fieF*). We exploit the natural iron storage function of ferritin to sequester iron and store it in the internal cavity to produce iron nanoparticles. Chimeric ferritin (AfFtn-m6A) has also been constructed to impart magnetotactic properties to *E. coli* where bacterial motility can be observed under the influence of an external magnetic field.

These iron nanoparticles with protein corona have also been produced *in vitro* in ambient conditions and their properties such as size, thermal stability and the effect of iron loading on the structure of the protein has been characterized. These ferritin protein nanocages, which can also be employed as carriers, have been loaded with photosensitizer acridine orange and have been shown to be cytotoxic upon exposure to light for potential use in photodynamic therapy as assistive surgery.

We envision a potential usage of the engineered nanoparticle-producing *E. coli* for magnetic resonance imaging of colorectal cancer. To complement the bacterium with specificity towards colorectal cancer, truncated membrane protein invasin was engineered to bind to colorectal cancer cells. The engineered bacterium was shown to bind to the β 1-integrins and internalized into the colorectal cancer cells within 2 hours of co-incubation. Multiple functionalities of tumor imaging and therapy have been engineered in *E. coli* and its derivative entities for the management of colorectal cancer by employing a synthetic biology based approach.

Chapter 1: Introduction

Synthetic Biology and metabolic engineering secured the second place in “The top 10 Emerging Technologies” in the World Economic Forum (WEF) held in February 2012. The reason for this choice as suggested by the forum “*The natural world is a testament to the vast potential inherent in the genetic code at the core of all living organisms. Rapid advances in synthetic biology and metabolic engineering are allowing biologists and engineers to tap into this potential in unprecedented ways, enabling the development of new biological processes and organisms that are designed to serve specific purposes—whether converting biomass to chemicals, fuels, and materials, producing new therapeutic drugs, or protecting the body against harm*”. Synthetic Biology is the design and construction of novel biological parts, devices, circuits, modules, chassis and systems, along with re-engineering of natural cellular components and tools to address various issues [1,2]. In one of the quirky studies which demonstrates the far-reaching potentials of this field in diverse applications, *E. coli* was engineered to see light [3]. The design consisted of a light sensing component which could sense the dark and respond by producing a dark pigment. *E. coli* cells were also programmed to identify the light-dark boundaries within a projected image with the help of genetic circuits [4]. The advent of synthetic biology has paved way to a more efficient production of fuels, chemicals and materials, in addition to creating smart circuits that respond to various signals allowing for a tight control and wide applications in health care [5,6].

Cancer is one of the leading causes of death worldwide according to the World Health Organization and the expected cases is set to rise by 70% in the coming two decades [7]. Among cancers, colorectal cancer is the third most leading cause of death in both men and women in the United States with 95,520 cases reported in 2017 alone [8]. Currently, cancer therapy majorly involves surgery, chemotherapy and radiation which lead to various side effects and complications. The main challenge in designing novel strategies to treat cancer is met with inability to differentiate between healthy and diseased tissue [9].

Magnetic Resonance Imaging (MRI) has been proposed as a tool in the early detection of colorectal cancer [10]. MRI is a non-invasive imaging modality that relies on the contrast differences between the soft tissue and the diseased tissue and this difference is enhanced by the use of contrast agents such as gadolinium [11]. However, concerns remain with the use of gadolinium due to the incidence of nephrogenic systemic fibrosis and retention in the kidney in patients with renal impairment [12]. Magnetic nanoparticles garner attention for use as contrast agents due to the significantly high relaxivity as compared to gadolinium based complexes [13]. Contrast agents loaded in nanocarriers such as microbubbles and liposomes were delivered to increase the concentration of the contrast agents [14,15]. The main properties required for the carriers of these contrast agents remain biocompatibility and stability in the physiological system [16]. Hence, replacement of the gadolinium contrast agents is much needed and this is achieved by the development of iron based contrast agents. In addition, for detection of colorectal cancer, MRI efficiency is

limited due to the bowel movement and has prompted for research to enhance visualization techniques.

Ferritin is a ubiquitous iron storage protein responsible for iron homeostasis in organisms except *Borrelia burgdoferi*, *Treponema pallidum* and *Lactobacillus plantarum* [17,18]. The three-dimensional structure of ferritin is highly conserved with 24 subunits self-assembling into a hollow cage like structure with 8 nm diameter. Ferritin acts as a size constraint reaction vessel for the production of monodispersed nanoparticles. Iron nanoparticles produced in ferritin have been shown to possess contrast enhancement properties making them an upcoming tool in the development of novel MRI contrast agents.

Past few decades stands witness to the various contributions made by nanotechnology to the field of oncology by the development of various liposome, lipid based nanoparticle therapeutics and microbial systems for the treatment of cancer [19]. In the development of therapeutic tools for the management of cancers in the GI tract (such as colorectal cancer), use of commensal bacteria has shown to pose no threat to the host and has come to be a novel therapeutic strategy [9]. Tumor colonization by intravenous administration requires high doses of bacterial introduction into the blood stream and hence, oral delivery of bacteria towards tumors was developed to achieve high colonization rates which would also minimize the systemic exposure [20-22]. Nanoparticle and microbial based cancer management seems to be an emerging field in the quest to find ideal cancer diagnostic and therapeutic entities.

This thesis aims to develop novel tools for therapeutic and diagnostic applications in managing colorectal cancer by using a synthetic biology based approach. Each chapter tackles a specific aspect related to the management of colorectal cancer. The first part of the work is based on the development of *E. coli* as a synthetic host for genetic rewiring and assigning novel functionalities for use in diagnostic applications such as MRI for the detection of colorectal cancer. The second part aims at therapeutic applications where protein nanocages produced in *E. coli* have been used as carriers of photosensitizers for effective treatment of cancer through photodynamic therapy. In the third part *E. coli* itself has been engineered to be directed towards colorectal cancer cells for selective uptake by the diseased cells. The specific objectives of each chapter are elucidated further.

Chapter 2 which consists of two parts discusses the various synthetic biology efforts made towards the synthesis of nanoparticles and other biomedical applications. Part A is a published version where it sheds light on the various engineered biological entities that are assigned novel functionalities in various healthcare applications including cancer diagnosis and therapeutics.

Chapter 3 describes the *in vivo* synthesis of iron nanoparticles in *Archaeoglobus fulgidus* ferritin nanocages using *E. coli* as the synthetic host. Synthetic biology based approach was employed for engineering *E. coli* to take-up excess iron by over expressing iron influx protein FeoB while the iron efflux was controlled by knocking out the efflux gene *fieF*. By engineering an *E. coli* strain capable of producing contrast agents *in vivo*, this commensal bacterium could potentially be employed as an MRI contrast agent to help in imaging of the colorectal tract.

The specific aims of this chapter are:

- Development of an iron accumulating strain of *E. coli* by over expressing the iron influx protein FeoB and knocking out iron efflux gene *fieF*.
- Estimating the iron loaded in the *Archaeoglobus fulgidus* ferritin expressed *in vivo* and characterizing the iron nanoparticles produced along with the engineered bacterium.
- Development and characterization of bacterium that is capable of responding to an applied external magnetic field.

Chapter 4 consists of the physico-chemical characterization of the ferritin nanocages synthesized in *E. coli*. The previous chapter discusses the iron loading achieved when the nanocages are loaded with iron *in vivo* while this chapter focusses on the characterization of these nanocages with controlled iron loading performed *in vitro*. Complete characterization of iron loaded ferritin nanocages would shed light on the ability of using these by themselves as a contrast agent in MRI.

The specific aims of this chapter are:

- Shape and size characterization of the *in vitro* iron loaded AfFtn and its mutant AfFtnAA.
- Studying the effect of iron loading on the secondary structure of the protein.
- Assessing the thermal and magnetic properties of ferritin nanocages with varied iron loading.
- Analysis of the cytotoxic effects arising from the iron nanoparticles in these cages towards colorectal cancer cells.

Chapter 5 elaborates on the use of ferritin nanocages for photodynamic therapy. AfFtn which has a hollow inner cavity allows for encapsulation of various drug molecules. Its properties of self-assembling in the presence of divalent metals provides a very simple method for loading drug molecules in its inner cavity. In this chapter photosensitizer acridine orange was loaded in ferritin nanocage to test its efficacy as a drug carrier and also evaluate the cytotoxicity of these nanocomposites towards colorectal cancer cells.

The specific aims of this chapter are:

- Loading photosensitizer molecule (acridine orange) in the inner cavity of AfFtn.
- Assessment of the photosensitization ability of acridine orange loaded in AfFtn.
- Studying the cytotoxicity of the acridine orange loaded colorectal cancer cell lines.

Chapter 6 consists of the work aimed at assigning specificity to the bacterium for selective uptake by colorectal cancer cells. To achieve this *E. coli* was engineered to selectively bind and respond to colorectal cancer cells for their specific uptake by the diseased cells.

The specific aims of this chapter are:

- Construction and expression of chimeric proteins in *E. coli* which can bind to $\beta 1$ integrins on colorectal cancer cells.

- Studying the binding of the engineered bacterium with colorectal cancer cells.
- Assessment of the internalization of the engineered bacterium into colorectal cancer cells.

Chapter 2: Literature Review

2.1 Part A: Biological Production of Nanoparticles

This section is published in “*Engineering Nanoparticle synthesis using Microbial Factories*” [23]

“Nanoparticles, the building blocks of nanotechnology, are particles with at least one dimension of less than or equal to 100 nm. Nanoparticles have potential applications in diverse fields that include targeted drug delivery vehicles, gene therapy and cancer treatment. Other applications such as antibacterial agents, DNA analysis, biosensors, separation science, magnetic resonance imaging (MRI) and nanogenerators, have been explored [24].

Although the size and shape of these particles might appear as merely physical, their complex effects on chemical properties and physical interactions are important in maintaining their functionality. For example, gold nanoparticles are reactive when they are less than 10 nm in size and on the other hand, particles with a small radius of curvature and angular shapes improve their catalytic properties [25,26]. Therefore, it is essential to synthesize shape- and size-controlled nanoparticles. However, this is met with multiple challenges. Nanoparticle synthesis is primarily categorized as either chemical or biological. Atomistic, molecular and particulate processing either in vacuum or in a liquid medium are applied in various chemical synthesis methods [27]. Many physical and chemical methods, which are extensively used, result in monodispersed nanoparticles but they require the use of harsh chemical stabilizers and capping agents. These chemical and physical

techniques are capital intensive and inefficient in energy usage [28]. In order to kinetically stabilize nanoparticles, stabilizers or capping agents such as surfactants, polymers, small ligands, cyclodextrins and polysaccharides are used [29]. The presence of capping agents may affect the functionalities of the nanoparticles and additional steps such as solvent washing, thermal annealing and UV-ozone irradiation are required to remove the stabilizers which could in turn alter the nanoparticle shape, size and stability [30,31]. Such synthesis procedures that use non-polar solvents and toxic chemicals on the surface to produce nanoparticles limit their usage in clinical fields. Hence, there exists a requirement for bio-compatible, clean, non-toxic and “green” methods of producing monodispersed, size-controlled nanoparticles. Despite the various disadvantages of physical and chemical synthesis methods, the time taken for production is shorter and involves fewer downstream processing steps in the purification.

Many microbes are naturally capable of producing nanoparticles either intracellularly or extracellularly when challenged with metal salts. The availability of various biotechnological tools, such as genetic and protein engineering, systems and synthetic biology, encourages the use of microbial systems to fine tune nanoparticle synthesis. This mini review highlights recent developments in the field of nanoparticle production by microbes (e.g. bacteria, cyanobacteria, yeast) and microbe-derived scaffolds, and identifies some challenges associated with the approach. There have been numerous reports on microbial synthesis of nanoparticles but very few studies have contributed to understanding the process and the mechanism of the syntheses. The knowledge is important to overcome the synthesis

bottlenecks and for the systematic engineering of microbes. The last part of the review sheds light on the future directions and highlights the importance of understanding the synthesis mechanisms for controlled production of nanoparticles.

2.1.1 Microbial production of nanoparticles

The concept of biological synthesis of nanoparticles was first reported in 1960 with marked progress being witnessed in the past 1.5 decades (**Figure 2.1**). Biological synthesis of nanoparticles refers to a wide array of biological techniques for producing nanoparticles by employing biological hosts including, but not limited to, bacteria, yeast, fungi, algae and plants. Successful microbial production of nanoparticles requires hosts that are tolerant to the products to be synthesized and the availability of molecular machineries. Proteins involved in the cell metabolism are found to be instrumental in the reduction of added metal ions and their conversion into metal nanoparticles with controlled size and morphology [32,33]. The main advantage of this approach is that it does not require any chemicals that can possibly be toxic and these reactions can occur at ambient temperature and pressure conditions.

Figure 2.1: Milestones in nanoparticle research (above) (above) [34-44] compared with that of genetic engineering (below) [45-51].

The biological process of nanoparticle formation in microorganisms can occur in two ways: intracellular (non-templated or templated) and extracellular (in the culture broth or adhered to the membrane) as demonstrated in **Figure 2.2**. In intracellular production, the cell culture is challenged with metal salt solution where the metal ions are transported across the cell membrane and nanoparticle formation occurs within the cell. Subsequently, the nanoparticles are recovered by lysing the cells and purified. In contrast, during extracellular synthesis the added metal salts are converted to nanoparticles either on the cell membrane or in culture broth. Recovery of the extracellularly produced nanoparticles will expectedly involve fewer downstream processing steps.

Figure 2.2: Different modes of nanoparticle biosynthesis by microorganisms. a) CdS quantum dots produced in genetically engineered *E. coli* [52]. b) Gold nanoparticles produced on the membrane of *Synechocystis* sp. PCC 6803 [53]. c) Silver nanoparticles produced by *Pseudomonas aeruginosa* SM1 [54]. d) Silver nanoparticles on plasmid scaffolds [44]. e) Magnetite nanoparticles produced in *Rhodospirillum rubrum* [55].

2.1.1.1 Bacterial production

Bacteria, owing to the ease of its manipulation and the availability of well-established genetic tools, are of particular importance in the field of nanoparticle synthesis. Easy maintenance and fast-growing time are added advantages. The first genetic engineering of bacteria for nanoparticle synthesis is reported by Chen and coworkers [41]. Genetically engineered *Escherichia coli* JM109 expressing phytochelatin synthase gene from *Schizosaccharomyces pombe* (SpPCS) along with modified g-glutamylcysteine synthetase (GSHI*) was used as a synthetic host to produce cadmium sulfide (CdS) nanocrystals. The GSHI* catalyzed the synthesis of glutathione (GSH), the precursor for phytochelatin, which in turn enhances the production of phytochelatin that serves as the capping agent for the CdS

nanocrystals. The strategy was adopted from *S. pombe* natural defense mechanism. Further improvement to the strategy shows that it is extendable to another strain, *E. coli* R189, resulting in the synthesis of uniform CdS quantum dot nanocrystals (3-4 nm).

In another study, *E. coli* was transformed with plasmids containing gene encoding foreign CdS-binding histidine-rich peptide (CDS7) [52]. High-resolution transmission electron microscopy, X-ray diffraction, luminescence spectroscopy, and energy dispersive X-ray spectroscopy were used to characterize the quantum dots and showed that the average particle diameter was 6 nm. Various alkaline earth, semiconducting, magnetic and noble metal nanoparticles have been synthesized using recombinant *E. coli* expressing PC from *Arabidopsis thaliana* and metallothione from *Pseudomonas putida* [56]. Some extremophiles, such as Antarctic bacteria, have natural resistance and tolerance towards cadmium and telluride, hence have been exploited for the synthesis of respective fluorescent nanoparticles [57]. However, the details of the mechanisms and the molecular machineries are not yet elucidated.

Silver nanoparticles produced from *Rhodobacter sphaeroides* are spherical and relatively mono-dispersed with average size of 9.56 ± 0.32 nm. The 6-h production time is an improvement compared to a few days in previous studies; hence, making it a rapid method to produce silver nanoparticles *in vivo* [58]. Some silver resistant *E. coli* strains contain CusCFBA silver/copper system which helps in the accumulation of silver nanoparticles in the periplasm [59]. Lin *et al.* have exploited such strain for anaerobic production of silver nanoparticles in the periplasm [60].

This process employs the use of oxidized metal ions as electron acceptors resulting in the generation of reduced metal nanoparticles with the help of multi-heme cytochrome-c [61,62]. Nitrate reductase (NapC) mutant of this strain ceased to produce silver nanoparticles establishing the role of cytochrome-c in the synthesis of silver nanoparticles. This study attempts to shed light on the mechanism of nanoparticle synthesis at the protein and metabolism level which deepens our understanding of the process (**Figure 2.3**).

Figure 2.3: A proposed model of biosynthesis of nano-Ag by periplasmic c-type cytochrome NapC in the silver-resistant *E. coli* strain 116AR [60].

Many studies have utilized bacteria to produce various kinds of nanoparticles but only a few have focused on understanding the synthesis mechanisms. Despite the exciting findings from these studies, limited information is available on the factors responsible for the synthesis. Understanding these systems is particularly important for controlled synthesis of unconventional nanoparticles, such as ruthenium and rhodium, and for further extrapolation into synthesis in easy-to-handle bacteria, such

as *E. coli*. *Pseudomonas aeruginosa* SM1 was used for intracellular production of lithium and cobalt nanoparticles [54]. The production was achieved without the addition of growth media, stabilizers, electron donors or pH adjustments while being performed at room temperature. The same strain was also shown to produce extracellular silver, palladium, iron, rhodium, nickel, ruthenium and platinum nanoparticles [54]. It is to be noted that this is the first report on rhodium and ruthenium nanoparticles production by living organism and nickel nanoparticle synthesis in bacteria.

Morganella morganii, a silver-resistant bacterium, was the first synthetic host reported to produce copper nanoparticles in aqueous phase [63]. Production of homogenous copper nanoparticles in aqueous phase is challenging due to the formation of copper oxide on the surface. The silver-resistant bacterium contains proteins that are similar to copper binding proteins; hence, it was envisaged that *M. morganii* is a suitable candidate for pure metallic copper nanoparticle synthesis. The resulting copper nanoparticles are 19 nm in size and devoid of copper oxide.

To add additional control to the biosynthesis of nanoparticles, biological templates have been explored to give better control in the shape and size distribution. Biological systems have natural ability for controlled deposition and structure of inorganic materials which has given rise to biomimetic approaches to synthesize inorganic nanomaterials. Protein shell with hollow cavities in the centre, such as cowpea chlorotic mottle virus (CCMV) capsids, ferritin and ferritin-like proteins, serve as a size-constrained reaction vessel for synthesizing inorganic materials with

controlled dimensions [64]. In addition, the protein surface provides a platform for surface modifications. An example of templated nanoparticle synthesis is the synthesis of 1D C-doped Fe₃O₄ nanoparticles inside self-assembled magnetosomes in magnetotactic bacterium, *Magnetospirillum gryphiswaldense* [65]. Characterization of the nanoparticles using field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) revealed nanoparticles of 50 nm size assembled into 1-2 µm long chains. Attempts to transfer the magnetosome biomineralization pathway from the magnetotactic bacterium, *Magnetospirillum gryphiswaldense*, for heterologous expression into another synthetic host, *Rhodospirillum rubrum*, were successful by incorporating *mamAB*, *mamGFDC*, *mamXY*, and *mms6* genes. The resulting magnetite nanoparticles were 24 nm in size surrounded by a protein shell [55]. The role of *mamO* gene was recently established in the formation of magnetic nanoparticles in magnetotactic bacteria by ruling out its long believed putative function of acting as a serine protease [66]. It was identified by X-ray crystallography and genetic analysis that the degenerate active site of the protein inhibits its protease activity. Also, a di-histidine motif which is surface exposed confers metal binding capability which is responsible for the initiation of biomineralization *in vivo*. A recent report suggests using *mms6* gene as a reporter for magnetic resonance imaging [67]. This was achieved by expressing the *mms6* gene in a mammalian cell line which in turn resulted in changes in magnetic resonance contrast due to formation of nanoparticles within the mammalian cells. The above study showcases the myriad applications possible by understanding the mechanism of nanoparticle synthesis.

2.1.1.2 Production of nanoparticles by photosynthetic microorganisms

Cyanobacteria, robust microorganisms which have the capability to adapt to extreme environmental conditions, are of particular interest as a synthetic host for nanoparticle production as they convert CO₂ to other forms of carbon catalyzed by sunlight [68]. The feature has implications on reduced cost for growth medium, hence lower production costs, and potentially reduced carbon footprint of the process. Silver and gold are the most reported nanoparticles synthesized using cyanobacteria as the host system.

Intracellular gold nanoparticles have been made using *Synechocystis sp.* PCC 6803 [53]. The nanoparticles of average size 13 ± 2 nm were found to localize at the cell wall, plasma membrane, and inside the cytoplasm. The study compared the gold nanoparticle synthesis to the metabolic activity of cyanobacteria namely, photosynthesis and respiration. It was observed that the production of nanoparticles was detrimental to photosynthesis in the presence of light but the same was not observed with respiration when cultured in the dark. The study also reported that photosynthetic electron transport in the thylakoid membranes played a key role in gold nanoparticle synthesis compared to the respiratory electron transport taking place at the cellular and thylakoid membranes. It is also speculated that the formation of gold nanoparticles within the cell wall of cyanobacteria may be due to the polyphosphates, polysaccharides and carboxyl groups present on the cell membrane which catalyze the reduction of gold ions. The findings are important for process development of gold nanoparticle production using cyanobacteria cultured in water under sunlight.

Sixteen different strains of cyanobacteria and microalgae were tested for their ability to produce silver nanoparticles of which fourteen were successful in the production [69]. Both cell extracts and extracellular medium (i.e. medium that had been used to grow the microbes) were capable of producing nanoparticles indicating that the extracellular medium contains excreted compounds responsible for the synthesis of nanoparticles of sizes 13-31 nm. Experiments showed that extracellular polysaccharides released by cyanobacteria and algae act as reducing agents in the nanoparticle synthesis. Interestingly, the extracellular medium failed to produce silver nanoparticles in the dark. In contrast, the washed biomass of a few strains retained the nanoparticle formation ability in the dark suggesting that light played a role in the process [70]. The study also demonstrated that C-phyococyanin, which is the blue colored accessory pigment produced by cyanobacteria, can reduce silver to form silver nanoparticles. Algal extracellular medium of *Chlorella vulgaris* when treated with chloroauric acid resulted in size and shape controlled nanogold crystal formation [71]. The protein, referred to as gold shape-directing protein, aiding in gold reduction was isolated and purified; and was further shown to produce triangular and hexagonal gold nanoplates.

A recent study employs genetically engineered micro-algae *Thalassiosira pseudonana* to attach IgG binding domain on bio-silica for its use as a cancer targeting moiety. IgG-bio-silica complex and chemotherapy drug (camptothecin and 7-ethyl-10-hydroxy-camptothecin) loaded liposomes were individually prepared.

Subsequently, the loaded liposomes were attached to the IgG biosilica complex and targeted towards cancer cells [72].

Cyanobacteria and algae seem to be promising hosts for nanoparticle synthesis. However, to increase the feasibility of using these reaction vessels for various applications, including nanoparticle synthesis, requires established genetic tools which is currently still lacking.

2.1.1.3 Production by yeast

Yeast, being a unicellular eukaryote, is an important model organism in molecular biology. Availability of genetic tools for manipulations makes it an interesting host to engineer for nanoparticle synthesis. Yeast has been employed for producing various metallic and non-metallic nanoparticles. Extracellular syntheses of gold, silver, and palladium nanoparticles are achieved using the yeast, *Hansenula anomala* [73]. Gold nanoparticles have been produced with and without the addition of stabilizers. The use of the G5 PAMAM dendrimer as the stabilizer gave bigger gold nanoparticles with average particle size of 40 nm whereas the average particle size without the addition of the stabilizer was found to be 14 nm. This generality of using bio-reductant was applied to silver and palladium resulting in nanoparticles of 35 nm as characterized by TEM.

Selenium nanoparticles which have a great potential as anti-cancer agents were synthesized using yeast *Saccharomyces boulardii* [74]. Extracellular synthesis of selenium nanoparticles was observed as the culture solution turned red from

colorless and the nanoparticle size averages 200 nm as revealed by TEM and dynamic light scattering (DLS) techniques.

The yeast, *Rhodotorula mucilaginosa* was employed as biofactory to produce copper nanoparticles [75]. Both, dead and live biomass could produce pure metallic copper nanoparticles and it was found that the dead biomass was more efficient in synthesis by producing the nanoparticles within 1 hour. It was speculated that the nanoparticle synthesis using dead biomass bypasses the toxicity barrier and the reducing enzymes form nanoparticles more efficiently. This provides an added advantage by removing the requirement for growth medium during synthesis.

Despite the availability of genetic tools and well-established methods of manipulation, genetic engineering in yeast to produce nanoparticles has not been well explored. There is immense scope in this area to identify limiting parameters leading to novel engineered pathway.

2.1.1.4 Other scaffolds

In addition to microbial synthesis and protein- templated synthesis of nanoparticles, cell-derived substrates such as plasmids and bacteriophages have been explored for nanoparticle synthesis. Plasmids derived from *Bacillus* host were employed as scaffolds to form silver nanoparticles [44]. Silver ions incubated with plasmids were photo irradiated under UV at 254 nm at room temperature and resulted in nanoparticles with an average diameter of 20-30 nm. The phosphate backbone of DNA being negatively charged, binds to the positively charged metal ions through electrostatic interactions. Photoirradiation with UV light led to nucleation of

nanoparticles on the plasmid which acted as a reducing agent, aiding in the formation of nanoparticles. This study shows that plasmids could be used not only as templates but also as reducing agents to drive the nucleation of nanoparticles.

Jeong *et al.* fused three glutamates on the N-terminus of the major capsid P8 of M13 bacteriophage [76]. Incubation with barium glycolates and subsequently with titanium glycolates resulted in calcination of this compound forming perovskite crystal structure while retaining the viral fibrous morphology (50-100 nm). Electrostatic interactions and hydrogen bonding led to the formation of barium titanate nanoparticles which were used as nanogenerators. This system could generate an electrical output up to ~300 nA and ~6V.

2.1.2 Discussion and future prospects

Various attempts to utilize microbes as factories to produce nanoparticles have resulted in nanoparticles of varied type, shape, color and size. Bacteria, owing to the ease of its handling and short multiplication time have been a favorite choice as a synthesis host. Thus far, the “green” methods in nanoparticle synthesis that have been explored are those available in nature almost without any modifications. Systemic design and production optimization for industrial level synthesis are challenged by the lack of understanding between causal mechanisms and the various interactions within the microbial system. For significant enhancement of the system, there is a definitive need for understanding the underlying mechanisms of biological nanoparticle synthesis. By establishing the pathways or the enzymes required for a particular kind of nanoparticle synthesis in a given organism, it is possible to

extrapolate this system to other hosts which would better suit the applications at hand.

Another important aspect to be taken into consideration is the availability of genetic tools for systemic manipulation of the genetic circuits in microorganisms. *E. coli* which has been a model organism for decades now is opted for genetic manipulation in the production of nanoparticles more frequently, due to ease of manipulation which is sufficed by the wide genetic tool set available for its engineering. More research needs to be focused on improving these genetic tools in other organisms such as cyanobacteria and unconventional yeasts which would aid in the employment of these non-traditional microorganisms for the synthesis of nanoparticles [77-79].

The challenges that remain to bring these processes to industrial scale are optimizing the production and minimizing the time required while choosing a suitable strain [80]. Development of microbial strains for industrial applications employing system-wide engineering, cellular metabolism optimization and product recovery optimization is a stand-alone research topic by itself [81]. Contemporary techniques in synthetic biology, systems biology and metabolic engineering serve as building blocks in industrial strain development. Strain improvement and controlled nanoparticle synthesis efforts should be brought together to design robust industrial strains capable of producing nanoparticles of desired shape and size.

The emergence of synthetic biology in this era will chart the development of this field in the next decades. Engineering non-native genetic circuits in yeast to produce

artemisinic acid and engineering *E. coli* to see light are some of the classic examples of the potential of this field [51,3]. Synthetic biology, which deconstructs biological systems into synthetic circuits and logic gates, will provide indispensable tools for tunable synthesis and optimization of nanoparticle synthesis in biological hosts. Synthetic circuits may be generated for the synthesis of nanoparticles with controlled size and shape. Understanding how the microbes react to stress and how the corresponding regulatory networks work will provide insights into metal toxicity and tolerance mechanisms. Integrating systems engineering approach in biology has paved way for the synthesis of small chemicals and similar advances are expected for the synthesis of nanoparticles.”

2.2 Part B: Nanotechnology and Synthetic Biology in Therapeutic Applications

2.2.1 Nanoparticles in healthcare applications

Nanoparticles are of particular interest owing to their size-dependent enhanced physical and chemical properties but recently the scenario has experienced a paradigm shift by looking at nanoparticles for various commercial explorations as in the case of nanomaterials for health care applications [82,83]. The projection of nanotechnology and its wide array of applications over time is highlighted in **Figure 2.4**.

Figure 2.4: Nanobiotechnology: A continuum of opportunity for nanotechnology in the life sciences (Reproduced with permission) [82].

A concise list of the applications of nanoparticles includes fluorescent biological labels, drug and gene delivery, bio-detection of pathogens, detection of proteins, probing of DNA structures, tissue engineering, tumor destruction via

heating, separation and purification of biological molecules and cells, phagokinetic studies and MRI contrast enhancement [24].

In specific, magnetic nanoparticles have a wide range of applications which include magnetic targeting for drug delivery and radionuclide therapy, contrast agents for magnetic resonance imaging, diagnostics, immune assays, cell separation and purification, RNA- and DNA- purification, cell adhesion, hyperthermia and magnetic ferrofluids for magneto-caloric pumps [84]. These magnetic nanoparticles have attracted interests in cancer therapies where they are employed to thermally kill hard to reach tumors by generating an alternative magnetic field [85].

2.2.2 Nanomedicine in cancer treatment

The widespread inclination to apply nanotechnology in cancer can be attributed to its myriad applications, such as diagnosis, imaging, drug delivery, vaccine development, miniature devices and the therapeutic nature of nanoparticles itself [86-89]. Currently, various therapeutic nanoparticles based primarily on liposomes, polymeric micelles and albumin nanoparticles have been approved for the treatment of cancer while several other nanoparticle- based treatment modalities are under clinical investigation which include hyperthermia, chemotherapy and radiotherapy, gene silencing, RNA interference and immunotherapy which are summarized in **Table 1** (reproduced from [19]) [90-109].

Table 1: Cancer therapy nanomedicines in clinical stage [19]. (Reproduced with permission)

Therapy modality	Generic name and/or proprietary name	Nanotechnology platform	Active pharmaceutical ingredients	Cancer type	Status
Chemotherapy: non-targeted delivery	Liposomal doxorubicin (Doxil)	Pegylated liposome	Doxorubicin	HIV-related Kaposi sarcoma, ovarian cancer, and multiple myeloma	Approved by FDA
	Liposomal daunorubicin (DaunoXome)	Liposome	Daunorubicin	HIV-related Kaposi sarcoma	Approved by FDA
	Liposomal vincristine (Marqibo)	Liposome	Vincristine sulfate	Acute lymphoblastic leukaemia	Approved by FDA
	Liposomal irinotecan (Onivyde or MM-398)	Pegylated liposome	Irinotecan	Post-gemcitabine metastatic pancreatic cancer	Approved by FDA
	Liposomal doxorubicin (Myocet)	Liposome	Doxorubicin	Metastatic breast cancer	Approved in Europe and Canada
	Mifamurtide (Mepact)	Liposome	Muramyl tripeptide phosphatidyl-ethanolamine	Nonmetastatic, resectable osteosarcoma	Approved in Europe
	Nab-paclitaxel (Abraxane)	Albumin NP	Paclitaxel	Breast, lung and pancreatic cancer	Approved by FDA
	SMANCS	Polymer conjugate	Neocarzinostatin	Liver and renal cancer	Approved in Japan
	Polymeric micelle paclitaxel (Genexol-PM)	Polymeric micelle	Paclitaxel	Breast cancer and NSCLC	Approved in Korea
	Liposomal cisplatin (Lipoplatin)	Pegylated liposome	Cisplatin	NSCLC	Phase III
	NK-105	Polymeric micelle	Paclitaxel	Metastatic or recurrent breast cancer	Phase III
	Liposomal paclitaxel (EndoTAG-1)	Liposome	Paclitaxel	Pancreatic cancer, liver metastases and HER2-negative and triple-negative breast cancer	Phase II
	Nab-rapamycin (ABI-009)	Albumin NP	Rapamycin	Advanced malignant PEComa and advanced cancer with mTOR mutations	Phase II
	CRLX-101	Polymeric NP	Camptothecin	NSCLC, metastatic renal cell carcinoma and recurrent ovarian, tubal or peritoneal cancer	Phase II
Chemotherapy: targeted delivery	MM-302	HER2-targeting liposome	Doxorubicin	HER2-positive breast cancer	Phase II/III
	BIND-014	PSMA-targeting polymeric NP	Docetaxel	NSCLC and mCRPC	Phase II
	MBP-426	TfR-targeting liposome	Oxaliplatin	Gastric, oesophageal and gastro-oesophageal adenocarcinoma	Phase I/II
	Anti-EGFR immunoliposomes loaded with doxorubicin	EGFR-targeting liposome	Doxorubicin	Solid tumours	Phase I
Chemotherapy: stimuli-responsive delivery	ThermoDox	Liposome	Doxorubicin	Hepatocellular carcinoma	Phase III
Chemotherapy: combinatorial delivery	Liposomal cytarabine–daunorubicin (CPX-351 or Vyxeos)	Liposome	Cytarabine and daunorubicin (5:1)	High-risk acute myeloid leukaemia	Phase III
	CPX-1	Liposome	Irinotecan and floxuridine (1:1)	Advanced colorectal cancer	Phase II

Nanoparticles are administered systemically for the treatment of tumors and they accumulate in the tumor environment due to the enhanced permeability and retention (EPR) effect which is attributed to the poor lymphatic drainage and leaky tumor vasculature [110-113]. EPR is largely influenced by the factors of systemic delivery of nanoparticles such as nanoparticle-protein interaction, extravasation into the tumor, blood circulation, perivascular tumor microenvironment, tumor penetration and internalization. The properties of the nanoparticles that affect its EPR effect are geometry, size, shape, surface features, porosity, and presence of targeting ligand [19].

2.2.3 Magnetic resonance imaging

A strong magnetic field applied to a sample aligns the protons in the sample giving rise to equilibrium magnetization along the longitudinal axis. The magnetic moments then flip away from the longitudinal axis with the application of a radiofrequency pulse. Once the radiation is removed, the magnetic moments of the protons relax to equilibrium and this is the relaxation time which has two components, longitudinal relaxation (T_1) and transverse relaxation (T_2) [114]. When the longitudinal relaxation is short, (short T_2 , the intensity of the signal is higher. T_2 is relative to how rapidly perpendicular magnetization plane loses coherence. T_2 is usually longer than T_1 and this difference in the relaxation time allows for the differentiation of tissues in images. The water protons present in the tissues give rise to the contrast in the MRI images. When the difference in relaxation between the diseased and normal tissue is marginal, significant difference in contrast is not

achieved in the image and hence contrast agents are required to amplify this difference [115,116].

Gadolinium which is the most widely used contrast agent is cytotoxic and tends to be retained in the spleen, liver and bone [117]. To surpass this toxicity, gadolinium is coated with large organic molecules which form a stable corona around gadolinium thus reducing the toxicity and increasing the elimination via kidneys [118].

2.2.3.1 Nanoparticles in MRI

Nanoparticles have gained impetus for use as contrast agents in MRI as they have several advantages compared to conventional gadolinium based contrast agents. The amount of the imaging agent in the nanoparticles can be controlled during synthesis. The surface of the nanoparticles can be modified to increase their circulation time or can be directed towards specific tissues [119,120]. Coated or encapsulated molecular constructs with metal payload have been constructed with micelles, liposomes, fluorinated nanoparticles, polymers, and dendrimers [121-123].

Manganese is also used as a contrast agent in MRI. Manganese chelates with DPDP, DTPA and porphyrin rings have been used for MRI contrast [124]. T₂ contrasting manganese oxides were incorporated in lipid bilayers which upon dissolution in the cells become T₁ contrast agents [125].

Iron oxide nanoparticles in the form of magnetite and γ -maghemite have been used as T₂ contrast agents. The upper size limit for these particles to exhibit paramagnetism is 25 nm and 30 nm respectively. These iron based nanoparticles are

stable under physiological conditions, have low toxicity and have high enough magnetic moments to achieve contrast [126]. However, these particles tend to aggregate naturally and hence surface modifications are opted. Frequently used polymers for coating are poly ethylene glycol (PEG), dextran, chitosan, alginate, polyvinyl alcohol (PVA), starch, albumin, poly ethylene imine, sulphonated styrene-divinyl-benzene, organic siloxane, and liposomes [127-129]. Mutant ferritin protein with ferrihydrite cargo was developed for MRI contrast which also provides an additional advantage of enhancement of the nuclear relaxation rate due to the presence of the protein associated water molecules [130,131].

Liposomes provide multiple advantages for their use in MRI contrast agents. Hydrophilic substances can be encapsulated in their aqueous inner core and hydrophobic compounds can be encapsulated in the bilayer [132]. Also, due to their good biocompatibility properties, they have been used as carriers for various gadolinium and iron based contrast agents [133,134].

2.2.4 Synthetic biology

Synthetic Biology, inspired by electrical engineering has its foundations laid by genetic circuits and biomolecular networks. Its main focus is to assign novel functions in microbial systems to render them capable of performing various tasks and solve many problems in diverse areas such as vaccine development, antibiotic resistance, microbiome engineering, regenerative medicine, cell therapy, infectious diseases and cancer therapy [135]. Synthetic Biology, as a field, gained impetus with the development of an engineered oscillator [136] and toggle switch [137] gene

networks in *Escherichia coli*. These pioneering studies have been followed by more complex and sophisticated gene networks which include counters, timers, clocks, logic processors, intercellular communication modules pattern detectors and mind-controlled systems [138-144,4]. These programmable regulatory networks are loaded onto cells thereby achieving the precise control of the phenotypic behavior. These advances are helping in shaping up the field and fine tuning its applications to specific and desired functional modalities. They have huge implications in medicine and healthcare segments such as personalized gene therapy.

2.2.4.1 Synthetic biology in cancer treatment

Current cancer therapies include surgery, chemotherapy and radiation which come along with a wide range of side effects and damage to healthy tissue. There arises the need for treatments that differentiate between healthy and diseased tissue. In this direction, bacteria have been engineered to invade cancer cells. This invasion was fine tuned to occur in a tumor environment by exploiting the hypoxic conditions of the tumor vasculature [145]. This was achieved by expressing invasin protein on bacteria which binds to the $\beta 1$ -integrins of the cancer cells, under the control of a hypoxia activated formate dehydrogenase promoter. Poor blood supply to the tumor vasculature limits the intravenous delivery of the engineered bacteria to the diseased tissue limiting the efficacy of the mode of delivery. A large number of bacteria needs to be administered intravenously making this approach inviable.

In another study, RNAi was used to knock down the gene CTNNB1 which is responsible for the initiation of the colon cancer when overexpressed [146]. This study loaded the cancer invading bacteria with shRNA which would block the

CTNNBI transcript, thereby stopping the translation of the gene. *In vitro* experiments showed knock down of the CTNNBI gene and *in vivo* experiments in immune deficient mice showed significant knock down in the tumor cells. However, concerns over the intravenous delivery of engineered bacteria remain due to lack of information on its effects and host immune response.

Engineered *E. coli* could be employed as cargo to deliver drugs or other protein molecules to tumor cells. In one study, *E. coli* was used as a tool to convert prodrug into an activated drug. Purine nucleoside phosphorylase which converts 6-Methylpurine-2'deoxyriboside into 6-methylpurine, a potent toxic drug, was delivered to tumor cells by invasive bacteria which resulted in the death of over 90% of the cancer cells in *in vitro* studies [147].

Adenovirus was designed to specifically kill cancer cells. This design strategy comprised of the viral replication genes (E1A and E4) under the control of human E2F1 gene regulatory region [148]. In normal cells the E2F1 gene is suppressed due to the presence of tumor suppressor proteins. Hence the delivered viral cargos would replicate and express genes only in tumor cells. A pox virus which exhibited replication in tumors selectively was chosen as a tool to engineer inactivation of the viral thymidine kinase gene while expressing human transgene for granulocyte-macrophage colony stimulating factor (GM-CSF) [149,150].

Chapter 3: *In vivo* Iron Nanoparticle Synthesis in Engineered *E. coli*

3.1 Introduction

3.1.1 Chemical and biological synthesis of iron nanoparticles

Chemical synthesis of iron oxide nanoparticles includes sonochemical, forced hydrolysis, electrochemical and sol gel methods which put into use harsh chemicals, high temperature and pressure conditions and yield nanoparticles in non-polar organic solvents which pave way for the need to develop green synthesis procedures [151, 152]. Iron oxide particle formation is naturally observed in many unicellular and multicellular organisms and is referred to as biomineralization. There are mainly two modes of iron biomineralization, biologically induced biomineralization (BIM) and biologically controlled biomineralization (BCM) [153]. BIM mode is a result of microbial metabolites released into the surrounding solution which react with the ions and lead to the nanoparticle synthesis. BCM is intracellular synthesis of iron nanoparticles which is under the strict control of the microorganism which is observed in magnetotactic bacteria. Iron sequestration into ferritin also falls under BCM.

3.1.1.1 Protein templates for nanoparticle synthesis

Biological systems could be exploited for controlled deposition and structure of inorganic materials which has given rise to biomimetic approaches to synthesize inorganic nanomaterials [64]. Synthesis of nanoparticles in size constrained environments have used, protein shell with hollow cavities in the centre, such as

cowpea chlorotic mottle virus (CCMV) capsids, ferritin and ferritin like proteins (**Figure 3.1**) [154]. These large cavities form novel reaction vessels for synthesizing inorganic materials with controlled dimensions. In addition, the protein surface provides a platform for surface modifications [155]. Chemical modifications could be performed on amino acid functional groups that are exposed to facilitate the attachment of an array of ligands and peptides. Protein engineering could pave the way for a wide array of applications by modifications on these biotemplates. In the case of ferritin, the natural process of biomineralization is exploited and it highlights the potential of being able to use biological molecules and their synthetic analogues for solid state reactions which is a very promising approach to nanophase engineering [154].

Figure 3.1: Schematic representation highlighting the use of ferritin for nanophase material synthesis. a) native reaction of iron oxide cores yielding iron sulfide b) manganese oxide core formation in apoferritin c) ion-binding and hydrolytic polymerization leading to uranyl oxyhydroxide formation [154].

3.1.1.2 Iron metabolism in *E. coli*

Iron is an essential element in all the organisms but it poses problems of poor solubility and toxicity. Hence, bacteria have evolved to possess various mechanisms which allow them to sequester iron from the environment, store it in storage proteins and also deport it when in excess, thus countering ill effects of excess iron by storing it in a non-toxic form in the cell. Iron, in the ferric form is internalized by the secretion of iron chelators into the environment which form a complex with the iron

and gets internalized. Iron, in the ferrous form enters the cells through the G-protein like transporters. *E. coli* contains approximately $10^5 - 10^6$ iron atoms per cell [156].

3.1.1.2.1 Ferric iron acquisition in *E. coli*

Bacteria secrete iron siderophores (iron chelators) that bind to the ferric iron and form complexes to solubilize the insoluble ferric to ferrous iron. These iron-siderophore complexes are recognized by the outer membrane receptors and are internalized by a process which depends on the energy transducing TonB-ExbB-ExbD system. These complexes are then transported into the cytosol with the mediation of the cytosolic membrane ATP-binding cassette (ABC) transporters [157].

3.1.1.2.2 Ferrous iron acquisition in *E. coli*

The iron transport system, Feo in *E. coli*, is responsible for the uptake of ferrous iron which is distinct from the siderophore dependent ferric iron uptake. *feoAB* genes were the first ferrous iron transport protein coding genes discovered in *E. coli* and are found to be conserved in many bacterial species [158]. The function of FeoB appeared to be of more importance in low oxygen conditions when ferrous iron pre-dominates the ferric form [159]. More details on FeoB is elaborated in later section. Also, extracellular ferric reductase activity has been observed in *E. coli* that converts the ferric iron to ferrous iron which could then be taken into the cell through the FeoB transport protein [160].

3.1.1.2.3 Iron storage in *E. coli*

Intracellular reserves of iron are deposited by the bacteria in their iron storage proteins [161] which is released and used as required by the cell. Three types of iron storage proteins found in *E. coli* are archetypal ferritin, heme containing bacterioferritins and the Dps proteins. The ferritins and the bacterioferritins are composed of 24 subunits which can accommodate approximately 2000-3000 iron atoms per 24-mer while Dps proteins are smaller storage proteins which are unique to prokaryotes, composed of 12 identical subunits with a capacity of approximately 500 iron atoms per 12-mer [162,163]. Ferrous iron is internalized into these proteins which is then converted to the ferric form by the ferroxidases and stored in the cavity. However, the main role of the Dps proteins in *E. coli* is to protect the cell from oxidative stress caused due to ferrous iron and hydrogen peroxide by the formation of the hydroxyl free radical rather than storage of iron [164].

3.1.1.3 Ferritin

Ferritin is a storage protein that sequesters iron in reduced form and stores it in its internal cavity in a non-toxic ferric form and modulates its release as required in the cell. Ferritin is a ubiquitous protein present in almost all life forms including bacteria, algae, higher plants and animals.

Typical bacterial ferritin cages are tetraeicosamers consisting of 24 identical subunits each of which is folded into a four-helix bundle and packed into a closed, hollow, spherical shell with octahedral (4-3-2) symmetry. Its inner and outer

diameter is 80 Å and 120 Å respectively and can store up to 4500 Fe³⁺ atoms in the crystalline mineral ferrihydrite form [165].

3.1.1.3.1 *Archaeoglobus fulgidus* ferritin (AfFtn)

Archaeoglobus fulgidus ferritin (**Figure 3.2**) is a hyperthermophillic sulfur metabolizing organism which exists mostly in turbulent hydrothermal environment where it encounters soluble Fe²⁺, H₂S, and oxygen. Avoiding the Fenton reaction and the spontaneous intracellular formation of ferric oxide or ferric sulfide, *A. fulgidus* rapidly sequesters iron into its unique ferritin quaternary structure.

Figure 3.2: Surface representation of octahedral common ferritin protein (left) and tetrahedral AfFtn protein (right) (Reproduced with permission) [130].

Unlike the commonly found ferritin with octahedral symmetry, *A. fulgidus* ferritin assembles into a unique, hollow, spherical shell with tetrahedral (2-3) symmetry with four large pores of approximately 45 Å diameter (**Figure 3.3**).

Speculations hold that these large pores may provide static, un-gated route for entry and release of iron. Also the overall larger dimensions of AfFtn lead to a higher iron loading capacity of approximately 7200 Fe per cage as compared to the common ferritin with a cage capacity of 4200 Fe [130].

Figure 3.3: Assembly of 24 subunits to form AfFtn protein (Reproduced with permission) [165].

3.1.1.4 Magnetite forming peptides

Iron oxide magnetic nanoparticles are produced in magnetotactic bacteria and various proteins associated with this mechanism have been elucidated [166]. One protein associated with the formation of iron oxide nanoparticles is amphiphilic mms6 with hydrophobic N-terminus and iron binding hydrophilic C-terminus [167,168]. A 12-amino acid C-terminus region of this mms6 protein, denoted as m6A was sufficient for the formation of magnetite crystals [169].

3.1.1.5 FeoB in *E. coli*

The first bacterial ferrous iron transport (Feo) system discovered was that of *E. coli* K-12 which is a facultative anaerobic gut commensal [170]. The *feo* locus consists of three genes *feoA*, *feoB* and *feoC* (**Figure 3.4**).

Figure 3.4: Organization of the FeoABC operon in *E. coli* (Reproduced with permission) [171].

FeoB which forms the major component of Feo is the main player in ferrous uptake by *E. coli*. It contains two components, a cytosolic G-protein domain (FeoB -N) and an integral-membrane domain (FeoB -C). When bound to Fe^{2+} , G-protein domain of FeoB hydrolyzes GTP to GDP, which is found to be very slow and implying that the G-protein domain of FeoB occurs mostly in the active GTP-bound form under normal conditions which is essential for efficient Fe^{2+} uptake. As observed in **Figure 3.5**, the two gate motifs (shown in light green) are possibly involved in Fe^{2+} translocation since both these gate motifs consist of a highly conserved Cys residue (shown in red, **Figure 3.5**) which is a ligand for Lewis acid including Fe^{2+} . The C-terminus consists of a transmembrane helix and a Cys-His rich region which may take part in metal-dependent control of FeoB-activity or Fe^{2+} translocation due to its potential Fe^{2+} binding ability [172].

Figure 3.5: Predicted schematic representation of ferrous iron uptake by Feo of *E. coli* (Reproduced with permission) [171].

3.1.1.6 Ferrous iron efflux F (*fieF*)

Cation diffusion facilitator (CDF) proteins are exclusive membrane bound, metal specific pumps which are present in all domains of life. They help detoxify the cations such as cobalt, nickel, copper, zinc, silver, cadmium and lead [173].

Iron which is the most abundant transition metal is essential at low concentrations but gets harmful at higher concentrations forcing the cells to establish intracellular homeostasis. Protein product of the gene *yiiP*, later renamed as *fieF* (**Figure 3.6**), belonging to CDF family, was identified to be responsible for the efflux of iron from the cells. Cells seemed to accumulate more intracellular iron when this gene was knocked out [174].

Figure 3.6: Structure of *fieF* gene product (PDB ID 2QFI).

3.1.2 Objectives of the chapter

This study which aims to synthesize iron nanoparticles in genetically engineered *E. coli* identifies the limiting factors of iron intake into the cells and overcomes the hurdles by overexpressing the required proteins which is hypothesized to enhance the iron intake and at the same time block the export of excess iron (**Figure 3.7**). This is a novel approach towards the synthesis of iron nanoparticles *in vivo*. This systematic engineering of the commensal bacterium *E. coli* is aimed towards its application as an MRI contrast agent for MRI of the colorectal tract.

Figure 3.7: Overexpression of AfFtn and FeoB along with the knock out of *fieF* in *E. coli*. Iron supplementation leads to formation of nanoparticles in the ferritin core.

The main objective here is to increase the *in vivo* iron loading capacity in *A. fulgidus* ferritin by expressing it in engineered *E. coli* BL21(DE3)C+RIL capable of over-accumulation of iron. This is achieved by overcoming the barriers which limit the accumulation of intracellular iron by over-expressing the iron influx protein *feoB* and deletion of the efflux protein *fieF*.

The specific objectives of this chapter include the generation of an *E. coli* knock out mutant $\Delta f i e F$ followed by over expression of AfFtn and FeoB in *E. coli* and $\Delta f i e F$. The engineered bacterium is supplemented with excess iron to form *in vivo* iron nanoparticles which are then characterized for their size and shape. The magnetic properties of the engineered bacterium are assessed and improved by further engineering the protein nanocage using magnetite forming peptide.

3.2 Materials and Methods

3.2.1 *E. coli* strains and plasmids

E. coli BL21(DE3)C+RIL with different plasmids was used as the host for the iron loading experiments. Various other strains and plasmids used for the knock out and construction of desired host are summarized in **Table 2**.

3.2.2 *fieF* gene deletion

Gene deletion strategy introduced by Datsenko and Warner was employed to knock out the gene *yiiP* (*fieF*) from *E. coli* BL21(DE3)C+RIL [175]. Using plasmid pKD4 as template, PCR primers (Primer 1 and Primer 2) with 50 bases homologous to the gene of interest (red) were designed to amplify FRT:kanamycin resistance cassette (kanR):FRT. The resulting PCR product, gene':FRT:kanR:FRT:'gene, was electroporated into strain BW25113 containing plasmid pKD46 which encodes an arabinose inducible Red recombinase. Transformants harboring deletion of the targeted gene were selected for kanamycin resistance located within the deleted gene. PCR (with Primer 3 and Primer 4) was used to confirm the deletion. The kanamycin cassette was removed by transforming in a temperature sensitive plasmid, pCP20, harboring yeast FLP recombinase gene leaving an 84 bp insert of a single FRT sequence at the site of deletion. Following are the primers used for the gene knock out.

Forward primer 1 – (*fieF* deletion fwd) - 70

CAGATGATTTGCTTCCGTTATACTAGCGTCAGTTGATAGCGGGAGTATT
TGTGTAGGCTGGAGCTGCTTC

Reverse Primer 2 – (*fieF* deletion rev) - 70

ATGGCGGTATTTTATACACAAAATGCGGGTCTGGCTCTCTTTTATACTG
AATGGGAATTAGCCATGGTCC

Primers for checking the correct insertion of cassette and checking deletion of the gene are below.

Forward Primer 3 (*fieF* del.check fwd) - 28

CAGATGATTTGCTTCCGTTATACTAGCG

Reverse Primer 4 (*fieF* del.check rev) - 29

ATGGCGGTATTTTATACACAAAATGCGGG

PCR mix (50µl reaction mix):

Component	Volume (µl)
ddH ₂ O	30
10X Pfu buffer with MgSO ₄	5
10mM dNTP	1
10 µM Forward Primer	5
10 µM Reverse Primer	5
Template	3
Pfu DNA Polymerase	1

Thermocycler Program:

95 °C	95 °C	55 °C	72 °C	72 °C	16 °C
3 min	30 sec	30 sec	3 min	10 min	∞
30 cycles					

3.2.3 Plasmids in *E. coli* BL21(DE3)C+RIL and *E. coli* BL21(DE3)C+RIL/ Δ *fieF*

Transformation protocol mentioned above is used to transform both the hosts, BL21(DE3)C+RIL and BL21(DE3)C+RIL/ Δ *fieF* with the plasmids pET-11a/AfFtn and pET-28a/*feoB* and also the empty vector pET-11a generating the bacterial strains

mentioned in **Table 2**. Antibiotic Ch denotes chloramphenicol, Amp denotes ampicillin and Kan denotes kanamycin

Table 2: *E. coli* strains with respective plasmids and antibiotic resistance.

<i>E. coli</i> Strain	Plasmids	Antibiotic
BL21(DE3)C+RIL	pET-11a	Ch, Amp
BL21(DE3)C+RIL	pET-11a/AfFtn	Ch, Amp
BL21(DE3)C+RIL	pET-11a/AfFtn, pET-28a/ <i>feoB</i>	Ch, Amp, Kan
BL21(DE3)C+RIL	pET-28a/ <i>feoB</i>	Ch, Kan
BL21(DE3)C+RIL	pET-11a/AfFtn-m6A	Ch, Amp
BL21(DE3)C+RIL/ Δ <i>fieF</i>	pET-11a	Ch, Amp
BL21(DE3)C+RIL/ Δ <i>fieF</i>	pET-11a/AfFtn	Ch, Amp
BL21(DE3)C+RIL/ Δ <i>fieF</i>	pET-11a/AfFtn, pET-28a/ <i>feoB</i>	Ch, Amp, Kan
BL21(DE3)C+RIL/ Δ <i>fieF</i>	pET-28a/ <i>feoB</i>	Ch, Kan

3.2.4 Bacterial culture

Iron loading experiments were conducted in 100 ml of the Lysogeny Broth (LB) containing 50 μ g/ml ampicillin, 30 μ g/ml kanamycin and 30 μ g/ml chloramphenicol as per requirement. An overnight culture in 5 ml LB was used for 1% inoculation of the 100 ml cultures. Induction was done using 0.5 mM IPTG as the plasmids contain a T7 promoter which is IPTG inducible, when the OD600 reached ~0.6. After 4 hours of induction, cells were loaded with 10 mM or 5 mM Fe₂SO₄.7H₂O and further grown for another 4 hours. Cells were then harvested by spinning down at 8,000×g for 15 minutes. Cells were then thoroughly washed with distilled water till the supernatant appears completely colorless which is approximately 5 to 6 times. The cell pellets were stored in the refrigerator at -80 °C till cell lysis.

3.2.5 Cell lysis and protein purification

The cell pellet was resuspended in 8ml of HEPES buffer (25 mM HEPES, 50 mM NaCl, pH 7.5). Cells were lysed using a Sonics Vibra Cell Ultrasonicator for 15 minutes at 5 sec pulse on and 5 sec pulse off at 37% amplitude. The lysate was spun down at 15000×g for 15 minutes to obtain the insoluble fraction in pellet. The supernatant was spun down again at 145,000×g for 1 hour to obtain membrane fraction in the pellet and soluble protein in the supernatant. This supernatant was heat treated 90 °C for 10 minutes and spun down at 145,000×g for 1 hour to remove any precipitated protein leaving behind semi purified soluble AfFtn.

3.2.6 Protein and iron quantification

Protein quantification

Protein estimation was done using the Thermo Scientific Pierce BCA Protein Assay kit. OD562 was measured using the VWR UV-1600 PC Spectrophotometer and the protein concentrations were estimated using the standards.

Iron quantification

The reagent is a mixture of 0.1 M HNO₃, 20 mM OBP (bathophenanthroline disulfonic acid), 0.46 M Tris-Cl buffer (pH 8) and 0.04M Sodium hydrosulphite. 75 µl of the protein sample was mixed with 75µl of the reagent and was allowed to stand at room temperature for 24 hours. Absorbance was measured at 538nm and 700nm using Spectramax M5 Microplate reader. OD538-OD700 was calculated which was plotted against the standards for a standard plot from which the iron content in unknown samples was estimated.

Iron per cage ferritin (24-mer) estimation

The protein concentrations (A) were obtained in µg/l and the iron concentrations (B) were obtained in µg/l. Iron per cage (24-mer) ferritin was calculated using the formula below.

$$\text{Iron per cage ferritin} = \frac{[\text{iron}](\mu\text{g/l})}{55.8} * \frac{490000}{[\text{protein}](\mu\text{g/l})}$$

3.2.7 SDS-PAGE

A 10% resolving gel was prepared and on top was a 4% stacking gel. Samples were mixed with equal volumes of the Laemmli dye (950 µl Laemmli + 50µl 2-mercaptoethanol). Samples were then heated in a 95 °C water bath for 10 minutes and then spun down at 10,000xg for 10 minutes. SDS-PAGE gels were loaded with the samples and Bio-Rad molecular weight ladder was used for size reference. Electrophoresis was performed at 100 V for 50 minutes. Gels were washed and stained using Coomassie Brilliant Blue Staining dye for 1 hour. Destaining was performed for 2 hours after which the gels were imaged using the imaging system.

3.2.8 Scanning electron microscopy

Sample preparation for Scanning Electron Microscopy (SEM)

Fifteen-ml bacterial cultures were induced with IPTG and iron loading where required, and the cells were harvested and washed thoroughly with water. Cells were then washed with 0.1 M phosphate buffer (PB). Cells were resuspended in a small quantity of 0.1 M PB and transferred to 1.5 ml micro-centrifuge tubes and allowed

to stand for half an hour. As much buffer is removed from the tubes, allowing sufficient quantity to prevent drying of the cells. The tubes were placed in a fume hood and 0.5 ml of the 2.5% glutaraldehyde in PB was added and allowed to react for 1 hour. Cells were then washed thrice, 2 minutes each with 0.1 M PB. Post fixation was done by adding 0.5 ml of 1% osmium tetroxide in water and allowed to react for 1 hour. Samples were then washed once with 0.1 M PB and twice with water. Dehydration was performed by treating with 25% ethanol and collecting the cells and the same procedure was repeated for 50%, 75%, 95% ethanol once and thrice with 100% ethanol to completely eliminate the water. 1:1 ratio of hexamethyldisilazane (HMDS) and ethanol was prepared and 0.5ml was added to each tube and allowed to stand for 15 minutes. It was then repeated twice with 100% HMDS and finally the pellet was allowed to dry overnight in the chemical fume hood.

Imaging using Field Emission Scanning Electron Microscope (FESEM)

Samples were loaded onto the double-sided carbon tape and were coated with Platinum for 200 seconds at 30mA current. Sample holder was fixed to the sample support and screwed tightly to not detach from each other. It was ensured that the liquid nitrogen tank was filled up, samples were applied into the Jeol JSM 6700F FESEM and the images were taken.

3.2.9 Magnetic measurements

Overnight cultures of bacterial samples were prepared, and 3% inoculation was performed in 100 ml M9 minimal media supplemented with required antibiotics

and grown at 37 °C for 4 hours. Protein expression was induced with the addition of 0.5 mM IPTG and continued to grow for a further 4 hours. Iron was supplemented to the cultures and half the culture (50 ml) was transferred into a falcon tube for growth in anaerobic conditions. The remaining half continued to grow in the conical flask. Anaerobic tube is placed in BD Gaspak anaerobic chamber (Becton Dickinson, USA). Both cultures are grown overnight at 20 °C, 200 rpm. The following day the pellets were collected and washed vigorously in PBS buffer and pelleted. Samples were freeze dried and the powdered form was used in the magnetic property measurement by using SQUID equipment (Quantum Design, USA).

3.3 Results and Discussions

In this section generation of an *E. coli* knock out mutant $\Delta fieF$ followed by over expression of AfFtn and FeoB in *E. coli* and $\Delta fieF$ is discussed. The engineered bacterium is supplemented with excess iron to form *in vivo* iron nanoparticles which are then characterized for their size and shape. The magnetic properties of the engineered bacterium are assessed and improved by engineering the protein nanocage using magnetite forming peptide and its magnetic properties are characterized.

3.3.1 *fieF* Knock-out Strain of *E. coli* BL21(DE3)C+RIL

The deletion of the ferrous iron efflux (*fieF*) gene (accession no. NC_000913) was successful using the Datsenko and Warner gene deletion method. Colony PCR on various colonies obtained shows the deletion of *fieF* gene while the control BL21(DE3)C+RIL shows the presence of *fieF* as can be observed from **Figure 3.8**. The deletion of the gene was also confirmed by sequencing using the primers flanking the gene on either side.

Figure 3.8: PCR results to check for absence of *fieF* and kanamycin cassette. Lanes 1- 7 show knock out of *fieF* while BL21(DE3)C+RIL shows the presence of *fieF*.

Once the knock out mutant *E. coli* (BL21(DE3)C+RIL/ Δ *fieF*) was obtained, its growth was monitored in comparison to *E. coli* BL21(DE3)C+RIL. The growth of the mutant was slightly slow as compared to the parent strain but overall the knock out did not seem deleterious to the cells. The growth curve obtained is presented in the **Figure 3.9**.

Figure 3.9: Growth curve of *E. coli* BL21(DE3)C+RIL and *E. coli* BL21(DE3)C+RIL/ Δ *fieF*.

3.3.2 Expression of *Archaeoglobus fulgidus* ferritin and *feoB*

AfFtn and *feoB* were expressed both in *E. coli* BL21(DE3)C+RIL and *E. coli* BL21(DE3)C+RIL/ Δ *fieF*. **Figure 3.10** shows the growth curve of AfFtn and *feoB* expression in *E. coli* BL21(DE3)C+RIL with and without induction (0.5 mM IPTG) and also with and without iron supplementation. It is observed that none of the conditions is lethal to cells and growth remained normal in all the conditions.

Figure 3.10: a) Growth curve of *E. coli* BL21(DE3)C+RIL with AfFtn and *feoB*, induced and non-induced, b) Growth curve of iron loaded *E. coli* BL21(DE3)C+RIL with AfFtn and *feoB*, induced and non-induced. Fe indicates 10 mM ferrous sulphate supplemented in the media.

3.3.2.1 SDS-PAGE analysis of gene expression

AfFtn and *feoB* were expressed in *E. coli* BL21(DE3)C+RIL and the protein purification steps were performed. Samples at every stage were analyzed by SDS-PAGE to check for soluble expression of AfFtn and expression of *feoB* in the membrane fraction. A ~73 kDa band represents the production of FeoB despite the fact that the theoretical molecular size of FeoB is 84 kDa [171]. Membrane proteins run at a different molecular weight in SDS as the proteins tend to unfold incompletely upon heating as a result of which the SDS detergent binds non-uniformly resulting in a phenomenon known as gel shifting. A ~73 kDa band was observed in the membrane fraction (P1) when the cells were induced with 0.5mM IPTG (**Figure 3.11**). The expression of FeoB seems to be lowered when the cells are loaded with 10 mM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$. This could possibly be due to reduced cellular capacity to over-express FeoB in iron over load conditions [156]. Another possibility is also the under-expression of native FeoB in *E. coli* in response to iron overload. It is known that the natural FeoB expression is tightly controlled by the Fur (Ferric uptake

regulator) protein and in the presence of excess iron, the intake is shut down by downregulating the natural FeoB expression [176,177].

However, this was not the case with AfFtn. A 20 kDa band corresponding to the monomer unit of AfFtn is observed when the cells are induced with IPTG and also remains unaffected when the cells are loaded with iron.

Figure 3.11: SDS-PAGE analysis of co-expression of AfFtn and FeoB in *E. coli* BL21(DE3)C+RIL (0.5 mM IPTG induction, 10 mM iron loading). P1 is the insoluble fraction, P2 is the membrane fraction and S is the final soluble fraction of the lysate.

3.3.3 Total protein obtained from cultures

Total protein that was obtained from various cultures was estimated using the BCA assay. From now onwards, DE3 implies *E. coli* BL21(DE3)C+RIL and $\Delta fieF$ indicates *E. coli* BL21(DE3)C+RIL/ $\Delta fieF$ for convenience. Two conditions were adopted for the iron loading experiments, one being 5 mM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ supplementation and other 10 mM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ supplementation. All the samples were induced with IPTG for protein production and purified protein was used for BCA assay. Samples supplemented with 5 mM iron (**Figure 3.12a**) and 10mM iron (**Figure 3.12b**) showed consistent protein production trend but the overall protein yield was higher in 5mM Fe supplemented samples as compared to 10mM. This signifies the stress levied on the cells by the excess amount of iron in the environment and this stress looks all the more prominent in the case of $\Delta fieF$ where protein yield is lower than its DE3 counterpart. Iron over-load on cells is known to have drastic effects on cell metabolism. When free iron reacts with superoxide and hydrogen peroxide in the cell, it leads to production of damaging hydroxyl radical reactive oxygen species [178]. In this system, the excess iron up taken from the medium is stored in the over expressed AfFtn and part of it that is not assimilated in the cage leads to damaging effects on the cell. These effects seem to be proportional to the amount of environmental iron and are visibly higher for higher iron supplementations (10 mM) as compared to 5 mM supplementation.

Figure 3.12: Total protein concentration (ppm) obtained from cultures supplemented with a) 5 mM and b) 10 mM ferrous sulphate .

The proteins in the controls containing pET-11a is expected to comprise of the housekeeping proteins. The energy to produce these proteins has been transferred to AfFtn and FeoB production in the other cases.

3.3.3.1 SDS-PAGE analysis of the semi-purified AfFtn protein samples

Purified protein samples from 10mM iron loaded cultures were subjected to SDS-PAGE analysis. SDS-PAGE of purified proteins from a) DE3 and b) $\Delta fieF$ strain is shown in **Figure 3.13**.

Figure 3.13: SDS-PAGE analysis of the semi-purified lysates showing AfFtn (20 kDa) protein expression from a) DE3 host and b) $\Delta fieF$ host. (+ indicates ferrous iron supplementation in the medium)

It is observed that both DE3 and $\Delta fieF$ expressing both AfFtn and FeoB together show a 20 kDa band, corresponding to AfFtn monomer unit. DE3

expressing AfFtn also shows a relatively thick 20 kDa AfFtn band (with and without iron loading) while $\Delta fieF$ expressing AfFtn contains the 20 kDa band only when loaded with iron. The reason for the unsuccessful expression of AfFtn in $\Delta fieF$ is not understood.

3.3.4 Iron concentrations from purified protein

Iron quantification was performed for the proteins that were purified from the cultures supplemented with either 5 mM or 10 mM iron (**Figure 3.14**). The purified proteins were brought to a final constant volume (5 ml) for the protein and iron quantification.

Figure 3.14: Total iron concentration (ppm) obtained from cultures supplemented with a) 5 mM and b) 10 mM ferrous sulphate.

From the SDS-PAGE in **Figure 3.13**, expression of a 20 kDa AfFtn band was observed in DE3+AfFtn+Fe, DE3+AfFtn+FeoB+Fe, $\Delta fieF$ +AfFtn+FeoB+Fe and $\Delta fieF$ +AfFtn+Fe. It is hence expected that high amounts of iron would be detected in these samples. However, high concentrations of iron were observed in

DE3+AfFtn+Fe, DE3+AfFtn+FeoB+Fe and $\Delta fieF$ +AfFtn+FeoB+Fe but not in $\Delta fieF$ +AfFtn+Fe. It is not clear why failed iron retention is observed in this sample. Iron supplemented controls (without the expression of AfFtn or FeoB) showed higher levels of iron in the semi-purified soluble protein fractions compared to the ones without iron supplementation suggesting that the natural phenomenon of iron internalization takes place upon excess iron availability only to a certain extent maintaining iron homeostasis [156].

3.3.5 Estimation of iron loading in AfFtn

The concentrations of protein and iron calculated in the preceding sections were converted to their molar concentration by dividing with AfFtn and iron molecular weight respectively. It is assumed here that the total protein concentration obtained from the semi-purified culture comprises totally of AfFtn. This assumption was made based on the clean bands obtained in SDS-PAGE of the semi-purified proteins. The ratio of the molar concentrations of iron to AfFtn gives the iron per cage (24-mer) which is shown in **Figure 3.15** (5 mM iron supplementation and 10 mM iron supplementation).

Figure 3.15: Iron per cage (24-mer) AfFtn when cells were supplemented with a) 5mM and b) 10mM ferrous sulphate.

The maximum average iron per cage obtained was 413 ± 50 Fe per cage AfFtn, in the case of $\Delta fieF$ +AfFtn+FeoB+ 10mM Fe, 351 ± 65 Fe per cage for DE3+AfFtn+ 10 mM Fe and 271 ± 67 obtained for $\Delta fieF$ +AfFtn+FeoB+5 mM Fe. Higher iron per cage values were obtained for a 10 mM iron supplementation as compared to 5 mM iron supplementation. These numbers are summarized in **Table 3**. By engineering *E. coli* to over express iron influx FeoB and shut iron efflux by blocking *fieF*, 4 times more iron could be accumulated in AfFtn expressed *in vivo*.

Table 3: Iron per cage (24-mer) AfFtn obtained in the engineered bacterial strains.

Strain	Producing Proteins	Fe per cage (24-mer)	
		5 mM Fe	10 mM
<i>E. coli</i> BL21(DE3)C+RIL	AfFtn	122 ± 32	351 ± 65
<i>E. coli</i> BL21(DE3)C+RIL	AfFtn+FeoB	215 ± 94	261 ± 3
<i>E. coli</i> BL21(DE3)C+RIL/ $\Delta fieF$	AfFtn+FeoB	271 ± 67	413 ± 50

3.3.6 Ferritin size measurements

To investigate the assembly of cages *in vivo*, Dynamic Light Scattering (DLS) was performed. This technique measures the hydrodynamic diameter of the

particles in solution by scattering pattern of the incident light. This technique is applicable only to spherical particles and hence can be used for the estimation of the diameter of AfFtn purified from cultures supplemented with ferrous sulphate. The DLS data (**Figure 3.16**) suggests that the cage diameter is approximately 15 nm which is in agreement with size of ferritin nanocage assembled *in vitro* [130]. As a positive control, cages assembled with 1200 Fe atoms is used to compare the size of the cages assembled *in vivo*. The size of the cages loaded *in vivo* is similar to the cages assembled *in vitro* (15 nm) indicating the correct assembly of the cages *in vivo*. Apo-AfFtn, which is purified from *E. coli* and is devoid of iron, exists as dimers with a hydrodynamic diameter of 8 nm as can be seen from the DLS result (**Figure 3.16**). However, a smaller cage size (12 nm) was observed for the control sample without iron supplementation which would expectedly have the lowest amount of iron taken up from LB medium. This observation is also in agreement with the reported literature that shows differences in AfFtn cage diameter with varied iron loading *in vitro* [179].

Figure 3.16: DLS showing 15 nm iron loaded AfFtn in *E. coli*/AfFtn, *E. coli*/AfFtn/FeoB and Δ *fieF*/AfFtn/FeoB and control *E. coli*/AfFtn/FeoB.

3.3.7 Examination of the cellular morphology at different iron loading

The expression of the membrane protein does not seem to affect the overall integrity of the cells but nevertheless a closer look at the cells indicate a certain degree of compromised cell wall (**Figure 3.17**). Rod shape of the cells is retained but membrane protein overexpression has disrupted the smooth cell wall structure. *E. coli* cells seem to have a fine and a smooth morphology but some cells seem to be extra-long. Cells overexpressing AfFtn and FeoB exhibit an approximate length of 2 to 4 μ m while *E. coli* BL21(DE3)C+RIL cells are of the size of 1 to 3 μ m with a few extra-long exceptions (**Figure 3.17**). Changes in cell morphology, either elongation or shortening, were observed in other reports due to membrane protein over-

expression [180,181]. The observations suggest that the cell division may be hindered.

Figure 3.17: High resolution Field Emission Scanning Electron Microscopy images of. a) *E. coli*/AfFtn/FeoB loaded with 10 mM iron, b) $\Delta fieF$ /AfFtn/FeoB loaded with 10 mM iron, c) *E. coli*/FeoB loaded with 10 mM iron, d) $\Delta fieF$ /FeoB loaded with 10 mM iron, e) *E. coli* BL21(DE3)C+RIL. Scale bar represents 1 μ m.

3.3.8 Construction of magnetic *E. coli*

Having established that the engineered bacteria can uptake and store higher amount of iron in AfFtn *in vivo*, to test whether the bacteria respond to an external

magnetic field, a 0.5 T magnet was placed under a well plate containing the iron supplemented bacteria but no movement in the bacteria was observed under the influence of the external magnetic field. Ferritins are known to store iron in the internal cavity of the protein in the form of ferrihydrite crystals with paramagnetic characteristics [182].

Iron oxides exist in different crystal structures with different structural and magnetic properties with the most abundant forms consisting of hematite (α -Fe₂O₃), magnetite (Fe₃O₄) and maghemite (γ -Fe₂O₃) [183,184]. (β -Fe₂O₃) which is paramagnetic at room temperature and epsilon (ϵ -Fe₂O₃) whose magnetic behavior is not fully understood, do not exist naturally and can be synthesized in the laboratory [185]. Hematite (α -Fe₂O₃) which is the most known form of iron oxide exists as a mineral in nature and has room temperature weak ferromagnetic or antiferromagnetic behavior [186]. Maghemite (γ -Fe₂O₃) is a ferromagnetic oxide which has thermal instability and is converted to hematite at temperatures above 673 K [187]. Magnetite (Fe₃O₄) presents ferromagnetic properties at room temperature [184]. Maghemite and magnetite can be magnetized easily at room temperature and show high magnetic response when an external magnetic field is applied [188].

The iron oxides in AfFtn core formed after *in vivo* loading are probably the non-magnetic forms. Here, to improve the magnetic properties of the iron core, we have explored the possibility of using an engineered ferritin AfFtn-m6A. m6A is a 12-amino acid peptide in the C-terminus of mms6 protein of magnetotactic bacteria. This 12-amino acid acidic peptide contains an iron binding site and is sufficient for

the formation of magnetite [169]. This peptide was attached to the C-terminus of AfFtn generating AfFtn-m6A fusion protein.

Expressing the engineered AfFtn-m6A in *E. coli* in LB medium did not help in generating bacteria capable of responding to an applied external magnetic field probably due to iron nucleation in aerobic conditions in the ferritin core. It is possible that non-magnetic iron oxide formation has taken place in the engineered AfFtn-m6A as well. Since, m6A peptide is derived from magnetotactic bacteria where, iron mineralization takes place in anaerobic conditions, we have tested growth in anaerobic conditions as well. Various growth conditions were tested for producing bacteria responsive to an external magnet. Bacteria expressing AfFtn-m6A was grown in M9 medium in aerobic conditions for 4 hours and iron supplementation was performed in anaerobic conditions which led to bacteria responsive to external magnet. This bacterium appeared dark expectedly due to the formation of magnetite inside (**Figure 3.18**).

Figure 3.18: A 0.5 T permanent magnet was placed under the bacteria supplemented with 10 mM iron. The bacteria could be seen aligning with the magnetic field of the magnet placed under it.

In **Figure 3.18**, where the bacteria were grown under anaerobic conditions after iron supplementation, the entire bacterial pellet was dark colored and all the bacteria was seen to move along the magnetic field. However, when the bacteria were further grown under aerobic conditions after iron supplementation, only a small fraction of the bacteria was found to be dark colored and also heavier than the remaining white colored bacteria. A 0.5 T magnet was introduced under the well containing the above bacteria. The dark colored bacteria aligned itself along the magnet field while the white colored bacteria did not respond to the external magnet and remained undisturbed in solution (**Figure 3.19**). This behavior is indicative of the differences in the kind of iron oxide formed in the ferritin core in aerobic and anaerobic conditions. The propensity towards formation of one type over the other is not yet understood and is subject to future investigations.

Figure 3.19: Magnet placed under *E. coli*/AfFtn-m6A grown in aerobic conditions in M9 medium supplemented with 10 mM iron 4 hours post induction and grown overnight at 20 °C.

3.3.8.1 Magnetic characterization of aerobically and anaerobically grown *E. coli*

External magnetic field (H) is applied on a material in one direction which is removed and then applied in an opposite direction to bring the magnetization to zero

and the magnetization loop traced is called as MH loop. The shape of the MH loop helps in the understanding of the magnetic properties of the material. If the loop is an open loop, the material is characterized as ferromagnetic. If the loop is a closed S, the material is superparamagnetic and a linear MH loop indicates paramagnetism.

For the magnetic characterization of the engineered *E. coli* expressing AfFtn-m6A, iron supplementation was done under aerobic and anaerobic conditions and the freeze dried pellet was characterized by i) induced magnetization as a function of the field and ii) measurements of induced magnetization (DC susceptibility) as a function of temperature after zero-field cooling (ZFC) and field cooling (FC) using superconducting quantum interference device (SQUID). **Figure 3.20** shows induced magnetization hysteresis loop for iron core formed in (a) aerobic conditions and (b) anaerobic conditions at different temperatures. The hysteresis loop was not observed for both samples at 300 K. However, the hysteresis loop was clearly seen below 10 K in samples where iron supplementation performed in anaerobic conditions indicating open hysteresis at low temperature. The hysteresis loops were found to be symmetrical about the origin indicative of the absence of antiferromagnetic component contributed by FeO or α -Fe₂O₃. For sample where iron supplementation was performed in aerobic conditions, open hysteresis was not observed implying the absence of magnetite formation in these conditions. Previous reports have shown that molecular oxygen is not required for the formation of magnetite by magnetotactic bacteria and most of the magnetotactic bacteria are anaerobes, facultative anaerobic microaerophiles or microaerophiles [189,38].

Figure 3.20: Induced magnetization hysteresis loop for iron core formed in a) aerobic conditions and b) anaerobic conditions at different temperatures. c) Magnetic hysteresis loop, saturation magnetization (H_c) and coercivity (M_s) of anaerobic iron supplemented sample obtained from the zoomed in image of (b). Green lines run through origin to show the symmetry about the axes. d) ZFC/FC curves for aerobic and anaerobic samples.

Figure 3.20c shows the magnetic hysteresis loop, saturation magnetization (H_c) and coercivity (M_s) of anaerobic iron supplemented sample. The measured higher values of coercivity ($M_s = 5800$ Oe) convey that the Fe_3O_4 nanoparticles are strongly ferromagnetic in nature.

To understand the magnetic behavior of the samples further, the temperature dependence of magnetization was measured in 50 Oe field between 2 K and 300 K, using the zero-field cooling (ZFC) and field cooling (FC) procedure. **Figure 3.20d** shows the ZFC/FC curves for aerobic and anaerobic samples. ZFC brought out a sharp transition at the blocking temperature (T_B), which is 10 K. Temperature

dependent magnetization under ZFC conditions shows rapid increase up to T_B . The ZFC and FC curves overlay on each other perfectly for anaerobic conditions, and the sharp transition at T_B could be attributed to the mono-dispersity in particle size of iron core. For the aerobic sample, the ZFC and FC curves do not show transition indicating the superparamagnetic nature of the ferritin core in these conditions. Anaerobic sample shows no magnetic hysteresis loop for higher temperatures, which is a transition from ferromagnetic state to paramagnetic state. In the case of aerobic samples, there is transition from super paramagnetic to paramagnetic state which can be deduced from ZFC curve. It is well documented in the literature that amorphous iron oxides show rather strong magnetic properties such as super-paramagnetic [190,191].

3.4 Conclusions

The *fieF* knock-out mutant of *E. coli* aiming to retain intracellular iron was successfully generated. The plasmids containing the genes AfFtn and FeoB were expressed and proteins were produced by the cells.

In vivo iron loading in *A. fulgidus* ferritin expressed in *E. coli* could be successfully accomplished by exploiting the metabolism in *E. coli*. Iron loading at 5 mM and 10 mM were compared for the maximum sequestration of iron into AfFtn. It was observed that 10 mM iron helped attain more iron per cage AfFtn as compared to 5 mM iron loading. The highest Fe per cage value (average) was obtained when the *E. coli* BL21(DE3)C+RIL/ Δ *fieF* /AfFtn/FeoB were loaded with 10 mM iron.

SEM images show rod shaped *E. coli* BL21(DE3)C+RIL and *E. coli* BL21(DE3)C+RIL/ Δ *fieF* also while expressing AfFtn and FeoB. Incorporation of the membrane protein FeoB does not seem to be affecting the overall morphology of the cells but resulted in compromised cell health.

Having engineered *E. coli* capable of increasing the *in vivo* iron loading in AfFtn, a variant of AfFtn with a magnetite forming peptide m6A (AfFtn-m6A) was generated to increase the magnetic properties of iron supplemented *E. coli*. Magnetic characterization revealed ferromagnetic properties when the *E. coli* was grown in minimal medium with iron supplementation carried out in anaerobic conditions. Further characterization and imaging of the magnetic *E. coli* is required to elucidate the kind of iron core and particle size formed *in vivo*.

Chapter 4: Metal core Formation and Characterization of *Archaeoglobus fulgidus* Ferritin (AfFtnWT) and its Variant (AfFtnnAA)

Parts of this chapter are published in “*Long-Range Tunneling Processes across Ferritin-Based Junctions*” [179].

4.1 Introduction

One of the most essential elements in the biological processes of almost all organisms is iron, owing to its multiple oxidation states which makes it an ideal biological cofactor [165]. The only known existing organisms that are iron independent are *Borrelia burgdoferi*, *Treponema pallidum* and *Lactobacillus plantarum* [17,18].

Ferritin is a ubiquitous iron storage protein responsible for iron homeostasis in organisms. Unlike bones and shells, where extracellular biomineralization takes place to provide protection and strength to an organic scaffold, ferritin protein sequesters metal ions within a single molecule of defined shape and size [182]. Ferritin sequesters the element iron and hence is a key player in iron metabolism. It acts as a buffer for the release and storage of iron from its internal cavity. Deregulation in its natural function, leads to a diseased state [192]. The three-dimensional structure of ferritin is highly conserved with 24 subunits self-assembling into a hollow cage like structure with 8 nm diameter [193]. Archetypal ferritin has a tetraeicosameric quaternary structure with the 24 subunits folded into four helix bundle which are arranged with a octahedral symmetry resulting in a

hollow protein with approximate outer and inner diameters of 12 nm and 8 nm respectively [194].

4.1.1 *Archaeoglobus fulgidus* ferritin and its variants

The marine archaeon *A. fulgidus* is a strictly anaerobic, sulphate reducing, hyperthermophilic organism. This extremophile thrives at the interface between frigid aerobic seawater and superheated anaerobic vent fluids that contain high concentrations of free ferrous iron [195]. Ferritin from *A. fulgidus* (AfFtn) has been structurally characterized and is found to be different from the typical ferritin [165]. The 24 subunits of AfFtn self-assemble to form a cage with tetrahedral symmetry unlike the octahedral symmetry exhibited by a typical ferritin. AfFtn exists as dimers in the absence of an iron core which is distinct from all other ferritins. The overall dimensions of AfFtn is larger with four large pores of 4.5 nm size. This difference in symmetry is thought to be due to the different amino acid residues in the E-helix which prevent a four-fold association of the subunits [196]. Each subunit is comprised of 5 helices and E-helix is the smallest of them. By changing the non-conserved residues (Lys-150 and Arg-151) to alanine the typical closed octahedral symmetry of ferritin was restored and this mutant is denoted as AfFtnAA (**Figure 4.1**). Iron binding kinetics were identical for both AfFtn and AfFtnAA indicating that the four large pores present in AfFtn are not involved in the ferrous ion access to the catalytic site. On the other hand, lowered reductive iron release was observed for AfFtnAA implying the role of iron release through the large pores [197].

Figure 4.1: Crystal structure of a) open pore, wild type *Archaeoglobus fulgidus* ferritin and b) its closed pore mutant AfFtnAA.

4.1.2 Nanoparticle synthesis in ferritin

Although ferritin naturally sequesters iron, various other metal nanoparticles have been synthesized in its cavity [198]. Europium, copper, cobalt, magnesium, manganese, gadolinium, nickel, cadmium, and zinc are examples of non-ferrous metal nanoparticles synthesized in horse spleen apo-ferritin [199-203].

Thermophilic ferritin, AfFtn was used for the synthesis of various metallic nanoparticles. Gold nanoparticles were formed in the cage by a templated synthesis mechanism in pre-assembled cages [204]. A manganese-ferritin composite which yielded 500 manganese atoms per cage was assessed for its suitability as an MRI contrast agent [205]. Cobalt (350 atoms per cage) chromium (2000 atoms per cage), and iron (7000 atoms per cage) nanoparticles were synthesized in the internal cavity of mutant AfFtn-AA [206].

4.1.3 Objectives of this chapter

While physicochemical characterizations of AfFtnAA has been reported, the molecular and cellular characterizations of the ferritin nanocages are still lacking. This chapter compares the size, shape, thermostability, secondary structure and magnetic characterization of the two formats of *A. fulgidus* iron loaded ferritin nanocages that are AfFtnWT and AfFtnAA at different iron loading. Cytotoxic effects arising from the iron nanoparticles in the cages towards colorectal cancer cells is also assessed.

4.2 Methods and Materials

4.2.1 Protein production

Overnight inoculum of AfFtn/pET-11a and AfFtnAA/pET-11a was prepared in lysogeny broth with 100 µg/mL ampicillin and 50 µg/mL chloramphenicol. Batch cultures of 6 L were inoculated with 3% overnight inoculum and antibiotics. OD600 of the culture was measure at regular intervals and induction was carried out with 1 mM IPTG at OD600 ~ 0.6. Cells were pelleted 4 hours post induction and the pellets were stored at -80 °C till cell lysis.

4.2.2 Cell lysis and purification

The cell pellet was re-suspended in 70 ml of Buffer A (25mM HEPES, 50mM NaCl, pH 7.5). Cells were lysed using a Sonics Vibra Cell Ultrasonicator for 15 minutes at 5 sec pulse on and 5 sec pulse off at 37% amplitude. Cell lysate was spun down at 25000xg for 30 minutes and the supernatant obtained was then heat treated at 90°C for 10 minutes which was then spun down at 25000g for 30 minutes. Ammonium sulphate was added to a final concentration of 500 mM and the protein was purified using hydrophobic interaction chromatography on AKTA-Explorer FPLC system (GE Healthcare). HiPrep 16/10 Phenyl FF (high sub) column was equilibrated with buffer A containing 500 mM (NH₄)₂SO₄. Elution fractions containing purified protein were pooled together and store at 4°C until further use. Concentration of the proteins was done as per requirement using 100 kDa molecular weight cut off filters and protein concentration was estimated as mention in the protein estimation section below.

4.2.3 Protein estimation using Bradford assay

Protein estimation was carried out using Bradford assay. Serial dilutions of BSA proteins were made and different dilutions of protein to be estimated were made. Bradford reagent (Bio-rad) was diluted to the working concentration and was added at a ratio of 20:1 to protein and incubated at room temperature for 5 minutes. Maximum absorbance was measured at 595 nm using Spectramax M5 and the protein concentrations were determined from the standard plot.

4.2.4 Iron loading in apo-AfFtnWT and AfFtnAA

Upon protein concentration calculation, the samples were diluted to 0.25 mg/ml in buffer A which is an equivalent of 0.5 μ M ferritin cage protein. Ferrous sulphate stock solution (100 mM) was prepared in 0.1% HCl. Desired molar ratios of the iron stock were added to the protein solution dropwise to attain different iron loading per cage ferritin. Samples were left at room temperature for 1 hour and then transferred to 4°C and stored over-night. Desalting and unbound iron removal was performed by using Amicon 100 kDa cut-off centrifugal filters and the samples were brought to a desired volume and then the protein concentration was measured again. 10ml of 0.1 mg/ml protein was sent for Inductively coupled plasma - optical emission spectrometry (ICP-OES) analysis to obtain the final iron concentration in the samples. Amount of iron loading per cage ferritin was then calculated based on the following formula.

$$\text{Iron per cage ferritin} = \frac{[\text{iron}](\mu\text{g/l})}{55.8} * \frac{490000}{[\text{protein}](\mu\text{g/l})}$$

4.2.5 Lyophilization of the purified protein

After purification and protein concentration determination, proteins were further concentrated to high concentrations (4 – 5 mg/mL) using 100 kDa cutoff filters (Millipore, U.S.A) by centrifugation (4000xg) to bring down the volume. Aliquots of 500 µl protein were made in 1.5 mL eppendorf tubes and were frozen at -80°C overnight. The following day, the vials were quickly sealed with a parafilm, pricked with a needle and lyophilized for 24 hours. The parafilm was removed and the vials were kept in the drying cabinet (Akarui Digi) maintained at 20°C till further use.

4.2.6 Dynamic light scattering

Protein size measurements were performed using dynamic light scattering in Zetasizer Nano ZS (Malvern Instruments). Protein samples at concentration of 0.5 mg/ml were spun down at 10,000xg for 10 minutes prior to measurements. Each sample was measured thrice in disposable cuvettes and each measurement was averaged out over 10 measurements.

4.2.7 Transmission electron microscopy

Proteins (0.05 ~ 0.1 mg/ml) were absorbed on the dark side of carbon-coated electron microscopy grids (Formvar carbon film on 300 mesh copper grids, Electron Microscopy Science) for 3 minutes followed by tapping out excess liquid on a soft wipe and then allowed to air dry for 5 minutes. The grids were then negatively stained with 1.5% uranyl acetate for 3 mins followed by tapping out of excess uranyl

acetate on a soft wipe and then allowed to air dry for 5 minutes. The grids were stored in a drying cabinet for 24 hours and images were obtained in a transmission electron microscope (JEOL JEM-1400) operating at 100 kV.

4.2.8 Thermogravimetric analysis

Lyophilized samples were added into the alumina pans (approximately 2-3mg). The pans were loaded onto a thermogravimetric analyzer (SDT Q600, TA instruments). The analysis was carried out over a temperature range of 30°C to 600°C in the presence of nitrogen at a rate of 10°C/min increase in temperature.

4.2.9 Circular dichroism

Far-UV spectra of AfFtn-WT/AfFtn-AA in water were analyzed using Applied photophysics circular dichroism spectrophotometer (Applied Photophysics Ltd, Leatherhead, UK). Measurements were carried out in Hellma GmbH&Co. quartz cells of path length 0.1 mm. The far-UV CD spectra between 190 nm and 260 nm were collected in 1 nm steps and 1s averaging time with 3 scans for every sample. Spectra represent the average of 3 scans from 3 different samples. By using the five points Savitzky-Golay smoothing filter in Chiralscan software (Applied Photophysics Ltd, Leatherhead, UK), baseline was subtracted from spectra and then further averaged and smoothened [207]. For accurate measurements, HT voltage lower than 1 kV over the range of wavelengths was ensured for adequate transmitted light to the detector. The following equation was used to convert the spectra to molar ellipticity (θ).

$$[\theta]_{molar\ ellipticity} = \frac{100 \times [\theta]_{deg}}{c \times l},$$

where $[\theta]_{deg}$ is the CD in degree, c is the molar concentration of protein, l is the cell path length in cm. By deconvoluting each spectrum in K2D2 (a web server for protein secondary structure estimation), proportion of secondary structure in the protein was estimated. K2D2 which is an artificial intelligence program uses a self-organizing map algorithm to obtain the correlations in data [208]. In the working wavelength range 190 – 240 nm, the far-UV CD spectrum gives the percentage of alpha helix and beta sheet in a protein [208,209].

4.2.10 Relaxivity measurements of AfFtnAA and AfFtnWT

Both variants of ferritin, (Fe4800)AfFtnWT and (Fe4800)AfFtnAA were prepared and the final concentrations of iron was estimated by ICP-OES. Serial dilutions of these protein samples were made as per known iron concentrations. For Nuclear Magnetic Relaxation Dispersion (NMRD) profiling, the T_1 and T_2 relaxation times were obtained by a mono-exponential fit of the signal intensities at 9.4 T by using a modified inverse recovery pulse sequence to account for radiation damping along with a Carr Purcell Meiboom Gill pulse sequence. A linear fit of the relaxation rate constant was constituted as a function of iron concentration from which the relaxivities r_1 and r_2 were calculated.

SQUID measurements were performed as described in section 3.2.9

4.2.11 Colorectal cancer cell proliferation

Colorectal cancer cell lines HCT116 and SW480 were seeded in 24 well tissue culture treated plates with 1ml of the growth medium and incubated at 37 °C, 5% CO₂ for 24 hours for the cells to adhere and reach a confluency of 75%. Varying amounts of AfFtnWT and AfFtnAA were added in the wells by replacing with fresh medium (500 µl) and the plates were incubated at 37 °C, 5% CO₂ for required time periods. 50 µl of the 0.15 mg/ml sterile resazurin solution was added to each well and cell proliferation was measured as a function of fluorescence units in a spectrophotometer (Spectramas M5) with excitation at 560 nm and emission at 590 nm. Resazurin, which is a cell permeable redox indicator, is reduced by the viable cells to a fluorescent, pink resofurin product [210].

4.2.12 Prussian blue staining

Colorectal cancer cell lines HCT116 and SW480 were seeded in 24 well tissue culture treated plates with 1 ml of the growth medium and incubated at 37 °C, 5% CO₂ for 24 hours for the cells to adhere and reach a confluency of 75%. Varying amounts of AfFtnWT and AfFtnAA were added in the wells by replacing with fresh medium (500 µl) and the plates were incubated at 37 °C, 5% CO₂ for required time periods. Wells were washed with DPBS 4 times to remove residual AfFtnWT/AfFtnAA. Cells were fixed with 4% PFA for 15 minutes and then washed with DPBS 3 times. Prussian Blue reagent was prepared by mixing equal volumes of freshly prepared 10% potassium ferrocyanide and 10% HCl. 200 µl of the reagent was added to each well and allowed to stand at room temperature for 20 minutes

followed by washing with DPBS thrice. Counter staining was performed by adding 200 μ l of 1% nuclear fast red solution, allowed to stand for 3 minutes followed by washing with DPBS thrice to remove residual nuclear fast red solution. 500 μ l DPBS was added to the well and images were taken in a bright field microscope.

4.3 Results and Discussions

This section compares the size, shape, thermostability, secondary structure and magnetic characterization of the two formats of *A. fulgidus* iron loaded ferritin nanocages that are AfFtnWT and AfFtnAA at different iron loading. Cytotoxic effects arising from the iron nanoparticles in the cages towards colorectal cancer cells is also assessed.

4.3.1 Protein purification and iron loading

In this chapter, proteins (AfFtn/AfFtnAA) were purified from bacterial cultures and iron loading was performed *in vitro*. This enables precise control over iron loading in the ferritin core. Typically, AfFtnWT and AfFtnAA bulk 6L cultures were prepared and the protein was purified using hydrophobic interaction chromatography (Appendix II, **Figure 10.1**). Fractions containing the ferritin were pooled and collected for protein estimation using Bradford assay. Upon protein quantification, the final protein concentration was brought to 0.25 mg/ml and followed by iron loading as per desired loading per cage.

For achieving a desired iron per cage ratio, corresponding molar ratios of iron (II) sulphate were added to the protein solution and purified by desalting to remove unbound iron. ICP-OES analysis was used to confirm the amount of iron loaded per cage (**Table 4**).

Table 4: Estimation of the iron loaded per cage AfFtn-WT and AfFtn-AA.

Desired iron loading	[Protein] ($\mu\text{g/L}$)	[Iron] ($\mu\text{g/L}$)	Iron per
AfFtn-WT			
600Fe/cage	23100	1377	523
1200Fe/cage	22000	2937	1172
2400Fe/cage	21500	5702	2328
3600Fe/cage	22500	9528	3718
4800Fe/cage	22400	12020	4712
AfFtn-AA			
600Fe/cage	20500	1377	589
1200Fe/cage	22300	2851	1122
2400Fe/cage	21500	6228	2543
3600Fe/cage	22700	8861	3427
4800Fe/cage	20900	10963	4606

4.3.2 Protein cage size measurements

After loading apo-ferritin with different amounts of iron, we checked for the size and formation of cages with DLS and TEM. DLS measures the hydrodynamic diameter of the particles in suspension and the diameter of apoAfFtnAA was found to be 7 nm, (Fe600)AfFtnAA was 13.5 nm, (Fe1200)AfFtnAA was 16 nm, (Fe2400)AfFtnAA was 16.6 nm, (Fe3600)AfFtnAA was 17 nm and (Fe4800)AfFtnAA was 17.9 nm and similar gradual increase in size of AfFtnWT cage was observed with increased iron loading (**Figure 5.2**) [179].

Figure 4.2: a) AfFtnWT (left) and b) AfFtnAA (right) size as measured by DLS for various iron loadings.

TEM images reveal the intact cage structure after iron loading which can be found in **Figure 4.3**. Uranyl acetate negative staining was used to image assembled ferritin nanocages loaded with 4800 iron atoms. Self-assembled cages of 15 nm diameter were observed from the TEM images.

Figure 4.3: Intactness of the cages as visualized by TEM with uranyl acetate negative staining. a) (Fe4800)AfFtnAA and b) ((Fe4800)AfFtnWT. Scale bar represents 50 nm.

4.3.3 Thermal stability analysis of the protein cages

The number of mass loss events in the thermal denaturation of a lyophilized protein is studied using thermogravimetric analysis. A constant heating rate is applied to the samples and the corresponding change in physical and chemical properties of the samples is obtained as a function of change in mass, temperature and time. The indicative physical changes are a result of fusion, crystallization, sublimation, vaporization, desorption, adsorption and absorption; while the chemical changes are due to chemisorption, decomposition, dehydration, oxidation, reduction and oxidative degradation [211]. In protein samples, the first mass loss step is due to the loss of water followed by degradation of the proteins [212].

Thermogravimetric analysis (TGA) was used to obtain the thermal stability information of AfFtn-WT and AfFtn-AA loaded with varying amounts of iron. Any changes in the degradation profile of the protein as a result of iron loading could be obtained here. The first derivative of the TGA curve (dm/dT) gives the Differential Thermo Gravimetric (DTG) curve. The change in mass with increase in temperature is represented in the TGA curve. The temperature at the maximum decomposition of the process is obtained from the minima in the DTG curve. The TGA curves obtained correspond to a typical TGA plot of a freeze-dried protein [213,214]. The mass loss between 40 °C to 200 °C which corresponds to the evaporation of the absorbed water due to evaporation is between 2% and 10% of the initial weight. This observation is normal for a freeze-dried protein [215]. The subsequent loss in mass that occurs between 200°C and 500°C is a result of decarboxylation, deamination and depolymerization due to the breaking of the polypeptide bonds. The degradation of

the cages occurs in steps which is indicated by the minima in the DTG curves (dashed line) in **Figure 4.4** and **Figure 4.5**. The temperature at which the mass of the sample decreases to 50% of the initial mass is the d.half temperature which is obtained at the intersection of the d.half line with the TGA plot (**Figure 4.4** and **Figure 4.5**). It can be deduced here that the degradation of the protein is not dependent on the iron loading. The bulk of the protein degrades between 280°C and 290°C.

Figure 4.4: TGA (solid) and DTG (dashed) plots of (a) apoAfFtnAA, (b) (Fe600)AfFtnAA, (c) (Fe1200)AfFtnAA, (d) (Fe2400)AfFtnAA, (e) (Fe3600)AfFtnAA and (f) (Fe4800)AfFtnAA.

Figure 4.5: TGA (solid) and DTG (dashed) plots of (a) apoAfFnWT, (b) (Fe600)AfFnWT, (c) (Fe1200)AfFnWT, (d) (Fe2400)AfFnWT, (e) (Fe3600)AfFnWT and (f) (Fe4800)AfFnWT.

4.3.4 Circular dichroism of AfFtn

Circular dichroism arises from the differential absorption of the two components of the plane polarized light and this is obtained for chiral molecules [216]. The peptide bonds in the protein absorb below 240 nm. The absorption of the aromatic amino acid side chains is between 260 and 320 nm while the di-sulphide bonds absorb around 260 nm [217]. In the far UV region, the different secondary structures in the proteins contribute to a characteristic CD spectrum. α -helicity of proteins can be estimated by the CD spectrum at 208 nm and 222 nm [218].

Increased iron loading in the core of AfFtn has resulted in increased diameter as seen in the DLS result in the earlier section. To study the effect of iron loading on the secondary structure of AfFtn due to the increase in size of the cage, CD technique has been used. From the CD spectrum of holo- (iron loaded) and apo-AfFtnAA (without iron), it can be observed that the α -helicity is higher in the apo-AfFtnAA as compared to the holo-AfFtnAA (**Figure 4.6**). This result is consistent with literature which showed similar differences in 24-mer and dimer ferritin [219]. The α -helicity of the proteins as calculated by CDDN software was 93% for (Fe600)AfFtnAA, 92% for (Fe1200)AfFtnAA, 93% for (Fe2400)AfFtnAA, 93% for (Fe3600)AfFtnAA, 97% for (Fe4800)AfFtnAA and 99% for apoAfFtnAA.

Figure 4.6: Circular dichroism spectra of holo (different iron loaded) and apo- a) AfFtnAA and b) AfFtnWT.

4.3.5 FTIR analysis of AfFtnWT and AfFtnAA

Peptide groups present in the proteins give rise to characteristic infrared absorption bands. Protein secondary structure information is obtained from its amide bond vibrations and amide I which is a result of C = O stretching, is used more often to deduce the secondary structure information. The amide I absorption lies between $1600 - 1700 \text{ cm}^{-1}$. The conformational sensitivity of amide bonds is governed by hydrogen bonds and transition dipoles [220]. IR absorption by the proteins in the range 1650 cm^{-1} to 1658 cm^{-1} is a result of the α -helical structures in the protein [221-223]. Second derivative of the FTIR spectra can provide qualitative information about the secondary structure of the protein [220].

To study the structural changes induced in the protein upon loading with iron, FTIR analysis was performed on apo-ferritin and different iron loadings of holo-ferritin AfFtnWT **Figure 4.7** and AfFtnAA **Figure 4.8**. The amide I bond peak is around 1647 cm^{-1} and the amide II bond peak is around 1552 cm^{-1} . From the secondary derivative curves of the amide I region, the α -helical structure peaks are

visible between 1650 cm^{-1} and 1658 cm^{-1} . The resolved peaks in this region are indicated in the respective graphs in **Figure 4.7** and **Figure 4.8**. The peak at 1658 cm^{-1} , shifts to 1659 cm^{-1} in 3600 Fe loading and 4800 Fe loading in both AfFtnWT and AfFtnAA. The peak at 1682 cm^{-1} is a result of the bends in the protein and is consistent across all the samples [220].

Figure 4.7: FTIR spectra of apo- and holo-AfFtnWT with different iron loadings (above). Second derivative of the FTIR spectra of all iron loadings in AfFtnWT (below).

Figure 4.8: FTIR spectra of apo- and holo-AfFtnAA with different iron loadings (above). Second derivative of the FTIR spectra of all iron loadings in AfFtnAA (below).

4.3.6 Magnetic characterization of AfFtnAA and AfFtnWT

The magnetic property of (Fe4800)AfFtnAA and (Fe4800)AfFtnWT was characterized by Nuclear Magnetic Relaxation Dispersion (NMRD). This experiment is performed with a series of serial dilutions of (Fe4800)fFtnWT and (Fe4800)AfFtnAA and their corresponding molar concentration taken on the x-axis. By plotting the linear fit relaxation rate constants as a function of iron concentration, the longitudinal (r_1) and transverse (r_2) relaxivities were calculated for both (Fe4800)AfFtnAA and (Fe4800)AfFtnWT (Figure 4.9).

Figure 4.9: a) longitudinal relaxation rate of (Fe4800)AfFtnAA, b) transverse relaxation rate constant of (Fe4800)AfFtnAA, c) longitudinal relaxation rate constant of (Fe4800)AfFtnWT and d) transverse relaxation rate constant of (Fe4800)AfFtnWT plotted against iron concentration. r_1 is the longitudinal relaxivity and r_2 is the transverse relaxivity.

The longitudinal relaxivity (r_1) obtained was $0.05 \text{ mM}^{-1}\text{s}^{-1}$ and $0.08 \text{ mM}^{-1}\text{s}^{-1}$ for (Fe4800)AfFtnAA and (Fe4800)AfFtnWT respectively and the transverse relaxivity (r_2) obtained was $5.35 \text{ mM}^{-1}\text{s}^{-1}$ and $10.5 \text{ mM}^{-1}\text{s}^{-1}$ for (Fe4800)AfFtnAA

and (Fe4800)AfFtnWT per iron atom respectively. AfFtnAA has been reported as a T_2 contrast agent in previous reports, however, AfFtnWT seems to have higher relaxivity as compared to AfFtnAA. This could be due to the large pores present on the protein which provide better access to the water molecules thereby enhancing the transverse relaxivity. The transverse relaxivity of (Fe4800)AfFtnWT composite obtained in this study ($r_2 = 50,000 \text{ mM}^{-1}\text{s}^{-1}$) is comparable to (Mn)AfFtnAA ($r_2 = 47,200 \text{ mM}^{-1}\text{s}^{-1}$) with a loading of 500 Mn per cage reported elsewhere [205].

For achieving significant contrast to noise ratio during imaging, relaxivities of particles should be around $1,000,000 \text{ mM}^{-1}\text{s}^{-1}$ [224]. By employing (Fe4800)AfFtnWT, this high relaxivity value can be reached with 20 molecules together. Hence by using iron nanoparticles produced in protein coronas such as ferritin, very high relaxivities can be achieved in concentrated areas.

4.3.7 Magnetic characterization of different loadings of AfFtnAA

The magnetic behavior of freeze dried AfFtnAA was investigated as a function of iron loading inside the ferritin cage by using superconducting quantum interference device (SQUID) at different temperature range. The magnetic properties of freeze dried holo-AfFtnAA and apoAfFtnAA powder samples were characterized by i) induced magnetization as a function of the field and ii) measurements of induced magnetization (DC susceptibility) as a function of temperature after zero-field cooling (ZFC) and field cooling (FC). Figure 4.10 shows induced magnetization hysteresis loop of different iron loaded AfFtnAA samples at 300K. The induced

magnetization values for biological samples are composed of the diamagnetic signal (protein shell), a superparamagnetic and a magnetically ordered phase. The hysteresis measurements were dominated by ferrihydrite cores of ferritin and show a non-saturating hysteresis up to high field and open hysteresis at small field (inset Figure 4.10b), suggesting the existence of a mixture of superparamagnetic and ferromagnetic core inside the ferritin at room temperature. The hysteresis loop is not observed when the loading increased above 1200Fe/cage. The shape of the curves for apoA₁F₁ sample indicates that the induced magnetization was dominated by the diamagnetic signal. The coercivity (H_c) of 220 Oe and 350 Oe was observed for 600Fe and 1200Fe loaded samples at room temperature respectively. The iron loading is critical for defining magnetically single-domain state for the samples (magnetic core size < 4 nm) below 1200Fe/cage and multi-domain state for the samples iron loading above 1200Fe/cage (magnetic core size 4–7 nm). The details of the ferromagnetic parameters are further elucidated from the low temperature measurements of the samples.

Figure 4.10c shows the magnetic hysteresis loop and Figure 4.10d shows saturation magnetization (H_c) and coercivity (M_s) of different iron loaded samples. The measurements show clearly that H_c and M_s depend on the iron oxide phase. The measured higher values of coercivity convey that the Fe_3O_4 and $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles are strongly ferromagnetic in nature. In general, the enhancement in coercivity and reduction in saturation magnetization could be due to strong dipolar interactions and other surface effect respectively, which can be distinct under certain conditions in the nano-regime.

Figure 4.10: Induced magnetization hysteresis loop of different iron loaded ferritin samples at 300K. d) Saturation magnetization (H_c) and coercivity (M_s) of different iron loaded samples.

As expected, the low-temperature curves show coercivity, and the maximum magnetization values in a 40 kOe field reach around 33 emu/g for <2400Fe loaded samples suggesting the existence of ~5 nm magnetite or γ -iron(III) oxide. The saturation magnetization is the lowest for 3600Fe and increases with further increase in iron loading indicative of phase transition or formation of magnetite core. The coercivity values saturate above 2400Fe loading indicating that ferromagnetic core formation reaches its maximum around 2400Fe loading and then single domain particles interact with each other. These interactions are strong at room temperature (no magnetic hysteresis curve) and weak at lower temperature.

To understand the magnetic behaviour of the samples further, the temperature dependence of magnetization was measured in 50 Oe field between 2K and 300K, using the zero-field cooling (ZFC) and field cooling (FC) procedure. Figure 4.11a shows the ZFC/FC curves for different iron loaded ferritin samples. ZFC brought out a sharp transition at the blocking temperature (T_B), which is increased from 9K to 15K with increasing iron inside the ferritin cage. Temperature dependent magnetization under ZFC conditions shows rapid increase up to T_B . Upon further increasing temperature, the magnetization decreases. The ZFC and FC curves overlay on each other perfectly for above 2400Fe iron loading, and the sharp transition at T_B could be attributed to the mono-dispersity in particle size of iron core Figure 4.11b. For samples with less than 2400Fe loading, the ZFC and FC curves does overlay indicating the partial formation of metal core inside AfFtnAA.

Figure 4.11: ZFC/FC curves for different iron loaded AfFtnAA. c) Temperature dependent magnetization in different iron loaded AfFtnAA. d) Hysteresis loop (zoomed in about the origin) of different iron loaded AfFtnAA.

The hysteresis loops were found to be asymmetrical with increasing iron loading up to 3600Fe loading, upon further increasing iron loading asymmetrical feature decreases indicating the presence of antiferromagnetic component via FeO or α -Fe₂O₃ (**Figure 4.11d**). Lower iron loaded samples show symmetric curve about the origin which rules out the formation of antiferromagnetic component. This result can be further confirmed by XPS data of AfFtnAA reported previously [179].

Thus, temperature dependent studies explain why field dependence magnetization at 300 K, which is well above T_B , shows no magnetic hysteresis loops for higher iron loaded samples, a transition from ferromagnetic state to paramagnetic state. It is well documented in literature that amorphous iron oxides show rather

strong magnetic properties such as superparamagnetic. The samples in the present investigation display a sharp transition from ferromagnetic to paramagnetic transition for higher iron loaded samples. The single domain magnetic properties can be seen at lower iron loaded samples.

4.3.8 Cytotoxic effects of AfFtnWT and AfFtnAA on colorectal cancer cells

To study the cytotoxic effects if any, of the iron loaded ferritin nanocages on colorectal cancer cells HCT116 and SW480, cells were incubated with varying concentrations of (Fe4800)AfFtn-WT and (Fe4800)AfFtnAA. Initially, low concentrations in the range of 0 to 0.1 μ M of AfFtn was tested and cell proliferation was measured at 4 hours, 8 hours and 24 hours and it was observed that the iron loaded ferritin did not show cytotoxic effects on the cancer cells as the viability of the control cells and treated cells were not significantly different even after 24 hours of treatment. Cytotoxicity experiments were conducted in triplicate samples three times and the average is presented here. **Figure 4.12** shows the results of lower concentration of (Fe4800)AfFtnAA (0 to 0.1 μ M (Fe4800)AfFtnAA) incubated with HCT116 and SW480. It is to be noted that the varied concentration is the concentration of the protein in μ M range and if this is to be compared with the concentration of iron, it would be 4800 times the concentration of protein approximately. The plots are obtained by normalizing the viability of treated cells against the control cells without protein treatment.

Figure 4.12: Cell proliferation of a) HCT116 and b) SW480 (right) treated with varying concentrations of (Fe4800)AfFtnAA in the lower protein range of 0 to 0.1 μM protein for 4, 8 and 24 hours. The viability of the control not treated with protein is 100% and the others are normalized relative to the control.

We proceeded to check the effect of higher concentration of protein on the colorectal cancer cells. The concentration of (Fe4800)AfFtnWT and (Fe4800)AfFtnAA was varied between 0 and 0.8 μM and the cell proliferation was tested 24 hours after incubation. With higher concentration of proteins, the viability decreased with increase in concentration of protein between 0 and 0.8 μM . The plots are obtained by normalizing the viability of treated cells against the control cells without protein treatment (**Figure 4.13**).

Figure 4.13: Cell proliferation of HCT116 and SW480 treated with varying concentrations of (Fe4800)AfFtn-AA (left) and (Fe4800)AfFtn-WT (right). a) HCT116 treated with (Fe4800)AfFtn-AA, b) HCT116 treated with (Fe4800)AfFtn-WT, c) SW480 treated with (Fe4800)AfFtn-AA and d) SW480 treated with (Fe4800)AfFtn-WT. Viability of untreated cells is set to 100% and other values are normalized against it.

It is observed that the cell density is reduced to half the control cell density around the highest concentration of protein used which is 0.8 μM . This high concentration of protein corresponds to even higher concentration of iron of approximately 3.8 mM. In other studies, HCT116 cells treated with high concentrations of iron nanoparticles lead to 80% decrease in viability after 24 hours [225]. Nanoparticles used in the study were 5 nm superparamagnetic iron oxide nanoparticles. 1 mg/ml nanoparticles did not have any significant cell cytotoxicity while 3 mg/ml concentration lead to 80% cell killing. However, this difference is

killing efficiency could be a result of the protective AfFtnWT/AfFtnAA corona on the surface of nanoparticles in our study. Similar observations were made when cerium nanoparticles were enclosed in horse spleen ferritin and its cytotoxicity was assessed with HepG2 cells where, free nanoparticles exhibited deleterious effect on the cells while protein trapped nanoparticles did not [226]. This is speculated to be due to the protective ferritin corona that keeps the nanoparticle away from interacting with various biomolecules in the cytoplasm. Platinum nanoparticles as well seemed to be less toxic to HepG2 cells when encapsulated in horse spleen ferritin due to the same reason [227].

HCT116 and SW480 cells were imaged under light microscope after performing prussian blue staining. Mixture of potassium ferrocyanide and hydrogen chloride is added to the fixed cancer cells and the reaction between the added reagent and ferric iron up taken by the cancer cells leads to the prussian blue color. As can be observed from **Figure 4.14**, **Figure 4.15**, **Figure 4.16** and **Figure 4.17**, (Fe4800)AfFtnWT and (Fe4800)AfFtnAA were taken up by the cancer cells. As the concentration of the incubated protein increases, the blue color intensity in the images increases indicative of higher amount of protein in the cells. Also, the viability data above suggests that concentrations higher than 0.4 μ M leads to decrease in cell proliferation which is also evident from the Prussian blue staining where blue color is observed all around the dead cells. Internalization of ferritin cages by cancer cells HCT116 is shown by using confocal microscopy in the following chapter. Prussian blue staining has been used to show internalization of AfFtnAA by macrophages in other reports [228].

Figure 4.14: HCT116 incubated with varied concentrations of (Fe4800)AfFtnAA for 24 hours stained with prussian blue.

Figure 4.15: HCT116 incubated with varied concentrations of (Fe4800)AfFtnWT for 24 hours stained with prussian blue.

Figure 4.16: SW480 incubated with varied concentrations of (Fe4800)AfFtnAA for 24 hours stained with prussian blue.

Figure 4.17: SW480 incubated with varied concentrations of (Fe4800)AfFtnWT for 24 hours stained with prussian blue.

4.4 Conclusions

AfFtnWT and AfFtnAA, over-expressed and purified from *E. coli*, were loaded with varying amounts of iron and their size was measured with DLS and the size of the cage was found to be around 15 nm on an average. However, an increasing trend in size of the cages (13.5 nm to 17.5 nm) from lower to higher iron loading was observed. TEM analysis showed intact cage structure in all the iron loadings.

Thermal stability analysis performed on freeze dried AfFtnWT and AfFtnAA showed thermal denaturation around 250°C for all the AfFtnWT and AfFtnAA loadings indicating non-dependence of thermal denaturation on iron loading. FTIR and CD analysis show that the secondary structure of the protein is independent of iron loading. The differences in the size of the cages with iron loading also do not seem to affect the secondary structure of the protein.

The magnetic characterization of ferritin yielded transverse relaxivity (R_2) 5.35 $\text{mM}^{-1}\text{s}^{-1}$ and 10.5 $\text{mM}^{-1}\text{s}^{-1}$ for (Fe4800)AfFtnAA and (Fe4800)AfFtnWT respectively. SQUID characterization on different iron loaded AfFtnAA shows a sharp transition from ferromagnetic to paramagnetic behavior for higher iron loaded samples. The single domain magnetic properties can be seen at lower iron loaded samples.

The cytotoxic effects of (Fe4800)AfFtnWT and (Fe4800)AfFtnAA was assessed on colorectal cancer cell lines HCT116 and SW480. Lower concentrations of the protein in the range 0 to 0.1 μM protein did not show cytotoxic effects on cell proliferation after 24 hours. However, at higher concentrations of protein (0.2 to 0.8

μM), the cell proliferation had reduced and half the cell density was reached with 0.8 μM of protein incubation. Ferritin internalized by HCT116 and SW480 was imaged using prussian blue staining after 24 hours of incubation.

Chapter 5: Ferritin Nanocages for Photodynamic Therapy

5.1 Introduction

5.1.1 Photodynamic therapy

Photodynamic therapy (PDT) is a more recent non-invasive modality in the treatment of tumors which uses a localized photosensitizer (PS) at the targeted tissue. This photosensitizer is activated with visible light which results in the tumor tissue photodamage and destruction. Various clinical trials have shown that PDT is a promising modality which is safe and effective in the treatment of tumors [229-231]. PDT has an edge over other cancer treatment methods currently used. This modality is not specific to the kind of tumors and hence it can be applied to any kind of tumors which is unlike chemotherapy or radiotherapy [232]. This outpatient procedure can be performed multiple times, if required, since it poses no cumulative toxic effects. PDT can be employed along with other surgical methods or chemotherapy for treating cancer to prevent the tumor metastasis [233]. This modality can be safely applied to elderly and also to people who are not fit for surgery due to its low risk profile [234].

However, there are several limitations associated with this treatment modality. It is not possible to administer high doses of the photosensitizers and light irradiate the whole body if the tumor has disseminated to various parts of the body in advanced stages. There is hospital and clinician resistance to new treatment methods alongside the difficulty in establishing optimum treatment variables. High costs are involved in the set up while there is a lack of a suitable and convenient light

source [230]. The efficacy of the drug is determined by the mode of drug delivery. Delivering the PS to target site while protecting it from the physiological environment is of paramount importance and novel nanocarrier carrier platforms to this end are currently under investigation in various studies [235].

5.1.1.1 Principles in photodynamic therapy

The illustration of the reactions in PDT is presented in **Figure 5.1** [236]. 1) Upon absorbing light of appropriate wavelength, the PS reaches its first excited state (S_1) from its ground state S_0 . 2) S_0 state can be re-achieved by emitting the absorbed energy from S_1 as fluorescence or by 3) internal conversion. 4) Also, possible by intersystem crossing is the conversion to relatively long first excited triplet state (T_1). 5) T_1 state can revert to S_0 state by the emission of phosphorescence. If this energy from T_1 state is transferred to the biological substrates or molecular oxygen, reactive oxygen species (ROSs) (1O_2 , H_2O_2 , $O_2^{\bullet-}$, $\cdot OH$) are released which result in cellular damage and death by necrosis or apoptosis. Apoptosis is initiated with an internal or external cellular signal which leads to caspase activation and eventually DNA fragmentation accompanied by cell shrinkage, pycnotic nuclear chromatin which collapses with nuclear membrane and the formation of apoptotic bodies by the breakage of cytoplasm and nucleus. On the other hand, necrosis hinders the ion homeostasis leading to water influx and eventual loss of membrane integrity [237,238]. Apoptosis is a preferred process as it can take place with lower irradiation as compared to that required for necrosis, with an anti-inflammatory effect with immune system stimulation. The type of cell death depends on various parameters such as the type of photochemical sensitization (type I or type II), cells and

photosensitizers and the amount of incident light [238,239]. For cell death by apoptosis, the induced damage should be greater than that required for cell repairing, but also mild enough for the production of energy required for the apoptotic pathway, else necrosis would take place [240]. Mitochondrial localization of PSs is shown to have higher apoptotic effects than localization in other cellular compartments [241-247].

Figure 5.1: Photophysical reactions in PDT [236].

5.1.1.2 Photosensitizers

Photosensitizers are molecules that absorb energy from the incident light source and transfers the energy to molecular oxygen which is converted to an activated form of oxygen called singlet oxygen (SO). This singlet oxygen is highly electrophilic, capable of oxidizing the biological electron rich double bonds. This phenomenon is associated with the cytotoxicity involved in PDT [231]. Photosensitizers are also involved in the electron transfer reactions which result in

radical induced damage. Both these processes in concert lead to the cell death associated with PDT [248].

PDT is one of the most important biomedical treatment modality which uses photodynamic action [236]. Light has been widely used for the treatment of various dermatological diseases in ancient civilizations but this treatment for cutaneous diseases gained impetus only at the beginning of the last century after the Nobel prize in this field was received by Niels Finsen. The use of photodynamic action dates back to the treatment of skin cancer using eosin along with light by Oscar Raab [249].

In 1960, the molecule hematoporphyrin derivative (HpD) was discovered and studied extensively by the Mayo Clinic which shed light and interest in PDT [250]. This soon accelerated by the huge study conducted by Dougherty involving 113 tumors from 25 patients. These tumors were treated with HpD which showed response in 98 tumors [251]. Photofrin, now a commercially available photosensitizer, which is a partially purified form of HpD, is the first photosensitizer to gain regulatory approval in over 40 countries for its use in cancer treatment in 1995.

Extensive research on different types of compounds lead to the identification of photosensitizers belonging to different classes such as chlorins, porphyrins, texafris, phenothiaziniums and phthalocyanins [252]. Second generation of photosensitizers are currently in clinical trials most of which are cyclic tetrapyrroles consisting of chlorin, porphyrin and bacteriochlorin derivatives (**Figure 5.2**) [236].

Drugs for PDT are administered systemically in most cases, while topical application and intra-tumoral injection have also been used [253,254].

Figure 5.2: Second generation photosensitizers [236].

5.1.1.2.1 Classification and characteristics of photosensitizers

Photosensitizers are broadly classified as belonging to the first, second or the third generation of photosensitizers [255]. HpD and Photofrin constitute the first generation of PSs. The second-generation PSs, reported in the 1980s, overcome the disadvantages of the first generation photosensitizers by having a better absorption profile and drifting towards non-porphyrin based photosensitizers. The third

generation which is the emerging class of PSs constitutes the second-generation PSs enclosed or coupled with carriers such as monosaccharides, peptides, low density lipoproteins, polymers, polymeric nanoparticles, liposomes, cholesterol and antibodies with targeting abilities [256-262].

Blood vessels in the tumor are significantly different from the normal vessels with extensive extravasation and angiogenesis, compromised vascular architecture, and diminished lymphatic clearance [263,264]. These properties which amount to a phenomenon known as enhanced permeability and retention (EPR) effect allow for the conjugated nano-sized PSs to be retained in the tumor vasculature. On the other hand, small molecules and PSs distribute themselves throughout the normal tissues due to lack of selectivity [265]. Nano-platforms for delivery also add advantages of high loading capacity, protect the PSs from degradation, increase circulation time while providing selectivity and controlled release [266]. Targeting and solubility of the PSs are the most important parameters to be considered for PDT treatment efficacies and these parameters may be enhanced by the use of nano carrier platforms for the delivery of the PSs [267,268].

The typical characteristics of PSs include 1) chemical purity in single species with synthesis and stability at room temperature. 2) Active only upon excitation with light and pose no harm in the absence of incident light. 3) Retained in tumoral regions and clean elimination from the body 4) Strong absorption of the incident light of longer wavelengths. 5) Photoreactivity should be excellent with higher triplet state yield and longer life at the triplet state which would increase the singlet oxygen

and ROSs generated and 6) Inexpensive commercial availability and ability to form formulations [269,270].

5.1.1.2.2 Acridine orange as photosensitizer

Acridine orange (AO), a weak basic dye, was extracted from coal tar over 100 years ago. Owing to its various biological activities, it has many applications as DNA/RNA fluorescent dye [271], photosensitizer [272], pH indicator [273], anti-tumor drug [272,274], anti-malarial drug [275], bacteria [276,277] and parasite detection [278], apoptosis [279] and sperm mobility [280].

The cytocidal effect of AO on osteosarcoma cells has been demonstrated earlier along with multi drug resistant osteosarcoma cells [281]. Within 24 hours of administration, swelling of cytoplasm and nucleus is observed and cell death occurs in 72 hours of AO-PDT. *In vivo* studies have also shown total tumor suppression in musculoskeletal sarcomas with AO-PDT [282]. AO is retained in the cancerous tissue for a longer time as compared to the normal tissue (**Figure 5.3**) [283]. AO fluoresces green when excited with blue light. This property allows for direct visualization of the tumors and can be used as an assisting aid during surgery [284,283].

Figure 5.3: Fluorescence image of the mouse osteosarcoma showing pulmonary metastatic lesions [283].

After the surgery with AO-PDT, the tumors are irradiated with X-rays (at a 5 Gy dose) which have a shorter wavelength and a higher energy. This seems to enhance the effect of AO-PDT [285]. It was later shown that X-rays could be used to irradiate PSs such as porphyrin [286].

5.1.2 Ferritin nano-platform for molecular entrapment

Ferritin nanocages have been exploited for the entrapment of cargos in several occasions. Propensity of horse spleen ferritin to disassemble at lower pH and reassemble at higher pH was exploited for loading various molecules such as neutral red dye, chemotherapeutic doxorubicin, gadolinium complex and PbS quantum dots [287-290]. Photosensitizers have been loaded into horse spleen ferritin using the pH modulation technique. Methylene Blue photosensitizer was encapsulated in horse spleen ferritin by raising the pH from 2 to 7.5 [291]. Zinc hexadecafluorophthalocyanine complex was loaded in human ferritin and used for photodynamic therapy in tumor models [292]. The pH driven re-assembly of the protein might pose undesired effects on the protein because of repeatedly traversing

the pI of the protein. Other methods of loading the photosensitizers are hence needed to overcome these difficulties.

Unlike other ferritins such as horse spleen or human ferritin that are more commonly used, AfFtn exists as dimers and in the presence of divalent metal atoms or high salt concentration, assembles to form 24-mer protein cage [165]. This unique feature of ionic strength mediated and divalent metal ions mediated assembly of the cages can be used for the entrapment of small molecules such as AO in the inner cavity of AfFtn nanocages.

5.1.3 Objectives of the chapter

To develop a novel third generation photosensitizer, this study attempts to encapsulate photosensitizer molecules in *A. fulgidus* ferritin. The property of AfFtn to exist as dimers in low ionic strength and in the absence of divalent atoms can be exploited for entrapment of acridine orange in its internal cavity either by introducing divalent atoms in a mixture of ferritin dimers and AO or by increasing the ionic strength of the same mixture. This is the first report of such photosensitizer encapsulation and use in photodynamic therapy. Encapsulation of PSs in protein cavities allows for modification of the peptides to target specific tissue. It also reduces the amount of PS that needs to be administered for photodynamic therapy. Low cost home lighting LEDs are used for the photosensitization of PS which reduces drastically the costs involved in setting up the photodynamic therapy system.

The specific objectives here include the encapsulation of photosensitizer acridine orange in the inner cavity of ferritin by using ionic strength mediated and ferrous ion

mediated entrapment methods followed by its characterization. The efficacy of photosensitization is measured by singlet oxygen generated and the cytotoxicity of the photosensitization is assessed on colorectal cancer cells.

5.2 Methods and Materials

Protein production and purification method is elaborated in the previous chapter. After purification, the proteins (AfFtnWT/AfFtnAA) were brought to a final concentration of 0.25 mg/ml).

5.2.1 AO loading in AfFtnAA and AfFtnWT

AfFtn protein was brought to a concentration of 0.25 mg/ml (0.5 μ M). A 1 mM Stock solution of acridine orange was prepared. For loading in AfFtnAA, 20 molar excess of AO was added followed by increase in ionic strength to 150 mM NaCl [(AO)AfFtnAA]. For loading in AfFtnWT, 20 molar excess AO was added to the protein solution, followed by addition of 600 molar excess iron sulphate hexahydrate [(AO,Fe)AfFtnWT] and increase in ionic strength to 150 mM NaCl [(AO)AfFtnWT]. All the samples were left at RT for 24 hours and were dialyzed for 2 days to remove unbound AO. The samples were then washed repeatedly and concentrated using 100 kDa MWCO centrifugal filters which would remove and residual dye or dimers. Concentration of the protein is then estimated using Bradford assay and the concentration of the dye was calculated by measuring the absorbance at 466 nm and compared with a series of known dye concentrations. Standard plot can be found in Appendix III (**Figure 11.1**).

TEM, DLS and CD were done as described in Chapter 4.

5.2.2 Photodynamic therapy – singlet oxygen generation and cell proliferation assay

Colorectal cancer cell lines HCT116 and SW480 were seeded in 96 well tissue culture treated plates with 1ml of the growth medium and incubated at 37 °C, 5% CO₂ for 24 hours for the cells to adhere and reach a confluency of 75%. The protein and AO concentration are measured in the freshly loaded AfFtn sample. AfFtn was added to medium to a final AO concentration of 50 nM and also medium containing 5 µM Singlet Oxygen Sensor Green (SOSG) (ThermoFisher Scientific) which binds to singlet oxygen generated after blue light treatment along with loaded AfFtn was prepared. Medium in the plates was replaced with the above medium. The plates are kept in the incubator 37°C, 5% CO₂ for 2 hours. Plates were then exposed to blue light for 30 minutes by removing every 10 mins to prevent heating up (no treatment on control plates), and the SOSG fluorescence was immediately measured (emission 504 nm and excitation 525 nm). Plates were then placed in the incubator at 37°C, 5% CO₂ for 24 hours and cell proliferation was measured by adding 10 µl of 0.15 mg/ml sterile resazurin solution and then continued to incubate for 2 hours followed by measuring the fluorescence output at 590 nm with an excitation at 560 nm in Spectramax M5 spectrophotometer.

5.2.3 Confocal imaging

Fluorescein isothiocyanate (FITC) dye was conjugated to the primary amines of (Fe600)AfFtnAA. This conjugation was performed by exchanging the buffer of (Fe600)AfFtnAA to 25 mM HEPES, 500 mM NaCl, pH 8 and concentrating using Amicon 100 kDa MWCO filters to a concentration of 5 mg/ml (~10 µM). Protein (1

ml) was taken in a 1.5 ml Eppendorf tube and 100 molar excess of FITC (1 mM stock solution in DMSO) was added. The tube was incubated at 4 °C for 2 hours. Conjugated protein was purified from unbound dye using PD10 desalting column (GE Healthcare) according to manufacturer's protocol. The degree of labelling was calculated according to FITC labelling calculation protocol (ThermoFisher Scientific) and was found to be approximately 10 dye molecules per cage. The size of the conjugated protein obtained after purification was also verified by DLS.

FITC conjugated AfFtnAA (final concentration 20 nM) was added to 75% confluent monolayer of HCT116 seeded on 8 well Ibidi microscopic chambers and incubated for 2 hours at 37°C, 5% CO₂. The wells were washed with DPBS thrice to remove residual AfFtn from the medium followed by addition of 100 nM LysoTracker red DND in McCoy's 5A medium. The plate was incubated for 2 hours at 37°C, 5% CO₂. The wells were then washed with DPBS twice. The nucleus was then stained with 200 µl of NucBlue (2 drops NucBlue in 1 ml PBS) (ThermoFisher Scientific) and washed thrice with DPBS. The cells were fixed with 4% PFA for 15 minutes and washed twice with DPBS. The chambers were then removed, and the glass slide was sealed with a coverslip using mowiol. Images were obtained in Zeiss LSM 800 confocal microscope at a 63x magnification.

5.3 Results and Discussions

The section discusses the encapsulation of photosensitizer acridine orange in the inner cavity of ferritin by using ionic strength mediated and ferrous ion mediated entrapment methods followed by its size characterization. The efficacy of photosensitization is measured by singlet oxygen generated and the cytotoxicity of the photosensitization is assessed on colorectal cancer cells by blue light treatment.

5.3.1 Acridine orange loading in ferritin

Acridine orange was loaded into both kinds of ferritin (AfFtnWT and AfFtnAA). Two different methods of loading, ionic strength mediated and ferrous ion mediated, were tested. AfFtnWT and AfFtnAA were incubated with 20 molar excess of acridine orange. For ferrous ion mediated encapsulation, 600Fe atoms were added right after adding AO [(AO,Fe)AfFtnWT]. For the ionic strength mediated encapsulation, AO was encapsulated both in AfFtnWT [(AO)AfFtnWT] and AfFtnAA [(AO)AfFtnAA] by bringing the ionic strength of the solution to 150 mM from 20 mM. The results for each kind of loading are summarized in **Table 5**. Maximum of 3 molecules of AO were encapsulated in AfFtnAA by increasing the ionic strength while 1 and 2 molecules of AO were encapsulated in AfFtnWT by increasing the ionic strength and by adding iron respectively. This result is an improvement compared to the methylene blue encapsulation in horse spleen ferritin where 1 molecule of methylene blue was encapsulated per ferritin cage [293].

Table 5: AO loading in AfFtn.

Ferritin - AO	AO/ cage ferritin
(AO)AfFtnWT	1
(AO,Fe)AfFtnWT	2
(AO)AfFtnAA	3

5.3.2 Size characterization of AO loaded ferritin nanocages

The size of the AO loaded ferritin nanocages were studied using dynamic light scattering, which confirms the average size of the particles to be around 15 nm which is in agreement with the size of the holo-ferritin nanocages (**Figure 5.4**). The presence of the caged structure was further confirmed by observing the particles in transmission electron microscope (**Figure 5.4**) which shows assembled ferritin nanocages. Both the techniques show that the protein nanocages are assembled into 24-mer cages.

Figure 5.4: TEM image of a) (AO)AfFtnAA, b) (AO)AfFtnWT, c) (AO,Fe)AfFtnWT; and d) DLS plot of apo and AO loaded in both variants of AfFtn.

5.3.3 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is a useful tool in studying the conformational changes that are induced in protein structures due to the binding with

small molecules [294]. It is the difference in the absorption measurement of left and right circularly polarized light arising from chiral molecules such as proteins. Small molecules that are bound to the protein give rise to an induced CD spectrum due to perturbations in its chemical structure and electron rearrangements. The distinct CD spectrum is a result of coupling of $\pi \rightarrow \pi^*$ transitions in each of the amide chromophores in the protein [295]. Proteins exhibit negative peaks with minima at 208 nm and 222 nm in the far UV range (250 – 190 nm) which is a characteristic of a predominantly α -helical protein [296].

The CD spectrum obtained for AfFtnAA is in **Figure 5.5**. The spectrum was obtained after the protein was in buffer with NaCl. As the chloride ions have high absorbance in the far UV region, the spectrum is shown from 190 nm to 260 nm [216]. The amount of α -helicity calculated using the CDNN software in AfFtnAA was 99%, (AO)AfFtnAA was 92%, and (Fe600)AfFtnAA was 93%. The α -helicity calculated for (AO)AfFtnWT, (AO,Fe)AfFtnWT, (Fe600)AfFtnWT and apoAfFtnWT was 99%. The α -helicity of the AO loaded cages is similar to Fe loaded AfFtn variants indicating that there is unlikely to be a significant change in tertiary or quaternary structure after AO loading.

Figure 5.5: CD spectrum of AO loaded in both variants of AfFtn. Samples include unloaded apo-ferritin, AO loaded AfFtn, (AO,Fe) loaded AfFtn and iron loaded AfFtn

5.3.4 Internalization of the ferritin nanocages

To establish whether AfFtnAA protein nanocages are internalized into colorectal cancer cells, AfFtn-AA was conjugated with FITC fluorophore. Conjugated protein was incubated with HCT116 cells for 2 hours and the fixed cells were imaged for the presence of the protein (**Figure 5.6**). The protein was internalized within 2 hours and some protein was also seen on the membrane of the cells. It was observed that the internalized protein was localized in the endosomes and lysosomes which is stained red. Other studies on uptake of horse spleen ferritin have showed co-localization of the cages in the lysosomes and cytoplasm of human breast cancer cells [297,298]. There are no previous reports of uptake studies of *A. fulgidus* ferritin into mammalian cells. It remains to be seen how the iron loaded ferritin nanocages are able to induce death in the tumor cell lines as the route of cell

death is important to understand the kind of cytotoxic stress (expression of proteins, and regulation of angiogenesis factors) levied on the tumors [240].

Figure 5.6: AfFtn-AA conjugated with FITC on N-terminus (green) incubated with HCT116 for 2 hours and stained for lysosomes (red) and nucleus (blue). a) Lysosomes red stain, b) green FITC-AfFtnAA, c) nucleus stained blue, d) merger of lysosomes and FITC-AfFtnAA and e) merger of lysosomes, FITC-AfFtnAA and nucleus.

5.3.5 Effect of light activation on the AO loaded cages with colorectal cancer cells

AO loaded in both variants of ferritin was incubated with colorectal cancer cell lines HCT116 and SW480 for 2 hours for the ferritin nanocages to be taken up by the cancer cells. The light irradiation leads to photosensitization and the generation of singlet oxygen which is monitored by the sensor green fluorescence (SOSG) output. It is observed that all the AO loaded ferritin nanocages gave rise to similar fluorescence output after 30 minutes of irradiation (**Figure 5.7**). Dye equivalent of 50 nM was maintained in all the samples. The baseline fluorescence of medium with and without SOSG reagent is the same. Activation of the PSs with blue light resulted in 60% increase in the fluorescence output which corresponds to the amount of singlet oxygen generated.

Figure 5.7: Singlet oxygen generation measured with SOSG sensor in a) HCT116 (left) and b) SW480 (right). Samples include AO loaded, (AO,Fe) loaded and Fe loaded AfFtnWT/AfFtnAA. Controls without blue light treatment are represented by black bars and blue light treated are in blue bars.

5.3.6 Cell proliferation after light treatment

Colorectal cancer cells were incubated with encapsulated AO for 2 hours followed by blue light treatment. Cell proliferation was measured 24 hours after blue light treatment by using the redox indicator resazurin assay. Decline in cell proliferation was observed in both cell lines, HCT116 and SW480. However significant decrease in cell proliferation was seen in HCT116 cell line with a 40% decrease in cell proliferation in (AO)AfFtnWT and 60% reduction in cell proliferation in (AO)AfFtnAA (**Figure 5.7**). The significant reduction was observed with only a single blue light treatment. Controls with no AO loading did not lead to cell death confirming that the cytotoxicity is a result of AO.

Figure 5.8: Viability of HCT116 after 24 hours w/wo blue light treatment. Samples include HCT116 alone, iron loaded AfFtnWT and AfFtnAA, AO loaded AfFtnWT and AfFtnAA, iron and AO loaded AfFtnWT and AfFtnAA, and a positive control of free AO. Blue bars indicate blue light treatment and white bars are not treated.

SW480 showed 25% decline in cell proliferation in (AO)AfFtnAA and around 10% decrease in proliferation with (AO)AfFtnWT and (AO,Fe)AfFtnWT

(**Figure 5.9**). Similar results were observed by Kim *et al.* when SW480 was treated with Pyropheophorbide-a as the photosensitizer [299]. Free acridine orange was shown to reduce the cell viability in osteocarcinoma cell line by 80% at a 1 $\mu\text{g/ml}$ concentration [300]. This concentration (1 $\mu\text{g/ml}$) is significantly higher than what is used in this study (50 nM, equivalent to approximately (13 ng/ml). The differences in the cell proliferation after PDT arise due to multiple reasons such as cell type, mode of delivery (free drug or nanocomposite). The route of cell signaling responsible for the induction of cell death defines the kind of cytotoxic stress (expression of proteins, and regulation of angiogenesis factors) levied on the tumors [240]. The route of cell death depends on the path of internalization of cages and hence the path of internalization of AfFtn in colorectal cancer cell needs to be explored further to understand the route of cell death.

Figure 5.9: Viability of SW480 after 24 hours w/o blue light treatment. Samples include SW480 alone, iron loaded AfFtnWT and AfFtnAA, AO loaded AfFtnWT and AfFtnAA, iron and AO loaded AfFtnWT and AfFtnAA, and a positive control of free AO. Blue bars indicate blue light treatment and white bars are not treated.

5.4 Conclusions

To develop novel protein enclosed third generation photosensitizers, acridine orange photosensitizer was successfully loaded in ferritin nanocages. The efficiency of loading in the cages was calculated and up to 3 molecules of acridine orange was loaded in the ferritin nanocages. Ferritin cage assembly was studied and characterized using DLS, TEM and CD which showed 15 nm, 24-mer assembled cages. Uptake of the ferritin nanocages by the colorectal cancer cells HCT116 was studied and the cages were seen to localize in the cytoplasm of the cancer cells as early as 1 hour after incubation.

Further studies were carried out on the photosensitization effect by the release of singlet oxygen and AO loaded cages contributed to 60% increase in the singlet oxygen generated. Cytotoxicity of the photodynamic therapy was assessed and the photosensitization lead to significant cell death in HCT116 as compared to SW480 colorectal cancer cell lines. The cytotoxicity of loaded AO was comparable to that of free AO indicating that encapsulation has no deleterious effects on photosensitization of AO. These results were also similar to the cytotoxicity of AO reported in other studies. Low cost home LED lights proved efficient in the photosensitization of AO paving way to significant decrease in the costs involved in photodynamic therapy applications.

AfFtn is a promising drug delivery platform with simple encapsulation strategy. The ease of loading the cargo along with the amenability for surface modifications to impart ligand specificity makes it an ideal choice as a delivery

vehicle. However, its mode of internalization into the cells needs to be assessed further to suit various applications.

Chapter 6: Engineering *E. coli* to target and respond to colorectal cancer

6.1 Introduction

Engineered bacterial systems that constitutively express targeting proteins that specifically bind to cancerous cells widely exploit the tumor microenvironment to induce the expression of therapeutic proteins to kill the cancer cells. This strategy although is very helpful doesn't prevent non-specific expression of inducible proteins as the expression depends on the microenvironment and not specific cancer cells. We, in this work envision to bridge this gap by developing a signaling circuit that transmits the external stimuli (binding to cancer cells) inside the engineered bacterium directing it to release the toxic protein.

To sense the external stimuli, bacterial two component signaling systems could be exploited as their natural functions include signal transduction. They possess a histidine kinase that senses the external stimuli and passes the signal inside the cell with the help of a cognate response regulator that in turn releases the target protein. The histidine kinase is engineered to possess $\beta 1$ integrin binding domain on the N-terminus. This chimeric protein is envisaged to bind to $\beta 1$ integrin expressed on colorectal cancer cells and transfer the signal intracellularly to release the toxic protein. The specific components and functionality of the bacterial two component signaling systems and design considerations for the sensing circuit are elucidated further.

6.1.1 Bacterial two component signaling system

Signal transduction in most prokaryotic and a few eukaryotic organisms involves phosphotransfer schemes comprising of two conserved components namely, a histidine kinase protein and a response regulator protein [301]. Bacteria sense the environment and respond to various external stimuli by well-coordinated signaling circuits. The primary mechanism of this perfectly coordinated response is via bacterial two-component signaling systems [302]. This system typically consists of a histidine kinase which is responsible for sensing the external stimuli and then pass on the signal to the response regulator that puts forth the required phenotypic changes. Upon sensing the external environment, the histidine kinase autophosphorylates a conserved histidine residue which in turn is transferred to the cognate response regulator [303]. This leads to conformational changes in the cognate response regulator which can bring changes in gene expression [304].

6.1.1.1 Histidine kinases in two-component signaling systems

Histidine kinases are dual functional proteins. When the external input signal is received they get autophosphorylated and the signal is transferred within the cell. In the absence of external input, they have a phosphatase activity on their cognate response regulators [305,306]. The kinase to phosphatase ratio defines the modular output ratio. These highly diverse kinases are able to transduce the signal through a combination of sensing, catalytic and auxiliary domains [307]. This modular nature of histidine kinases allows them to be adapted to specific requirements of the signaling systems [308].

There are two highly conserved domains in all histidine kinases. The first one is the histidine phosphotransfer (DHp) and dimerization domain which contains the conserved histidine residue responsible for both autophosphorylation and phosphotransfer reactions (**Figure 6.1**). The second domain is the catalytic and the ATP-binding domain (CA). Histidine kinases often contain one or more additional domains towards the N-terminal of the DHp domain [309]. These additional domains contain the transmembrane part of the protein and the signal recognition domains that usually span in the periplasmic or the extracellular part of the protein [310]. Signal recognition domains are the more variable domains as compared to other domains in histidine kinases. More often than not, there exists another domain between the transmembrane domain and the DHp domain which is mostly a HAMP, PAS or GAF domains, responsible for the relay of the external signal from periplasm to DHp or CA [311,312]. The activity of the histidine kinase is controlled by the input signal received by the sensing domain. The autophosphorylation is ATP dependent and occurs at the conserved histidine residue. This reaction is a bimolecular reaction that occurs between two homodimers, where one monomer catalyzes the phosphorylation reaction at the conserved histidine residue of the second monomer [313-318].

Figure 6.1: The schematic of Tar-EnvZ chimeric protein, Taz showing EnvZ histidine kinase domain. TM is the transmembrane; DHp is a dimerization histidine phosphotransfer domain and CA is a catalytic ATP-binding domain.

There are variations to the typical histidine kinases which include phosphorelay. These pathways contain a hybrid kinase which is a fusion of a receiver domain to the C-terminus of the histidine kinase [319]. The autophosphorylation is followed by an intramolecular phosphotransferase of the phosphoryl group to the receiver domain which is then transferred to a histidine phosphotransfer and eventually to a response regulator resulting the output response. Hybrid kinases constitute a quarter of the histidine kinases suggesting a widespread prevalence of phosphorelays [320].

6.1.2 EnvZ-OmpR two-component signaling system

EnvZ-OmpR is a well characterized two-component signal transduction system [321-323]. This system regulates the expression of OmpF and OmpC which are outer membrane porins that sense the osmolarity changes [324,325]. In this two-component signaling system, EnvZ is the transmembrane histidine kinase with a cognate response regulator OmpR. OmpR, a transcription factor regulates the expression of ompC and ompF [326,327]. The cytoplasmic domain of EnvZ can autophosphorylate via a conserved His residue by phosphate transfer by ATP [328,329]. This phosphoryl group is then transferred to a conserved Asp on generating phosphorylated OmpR [330]. EnvZ also possess phosphatase activity on OmpR [331]. Phosphorylated OmpR regulates the expression of OmpC and OmpF hence responding to the osmolarity changes. This system has previously been engineered to sense and respond to aspartate instead of its natural osmolarity sensing ability by changing the extracellular ligand binding region of the protein [332]. The cytoplasmic domain of EnvZ could hence be exploited to make chimeric proteins with signal sensing domains on N-terminus for signal transduction.

6.1.3 VirA-VirG two-component signaling system

Agrobacterium tumefaciens is a soil bacterium that is able to induce crown gall tumors in various hosts [333-335]. This tumorigenic ability is controlled by a two-component signal transduction system VirA/VirG [336]. VirA, which is the sensory kinase recognizes and responds to the hotness molecule acetosyringone and induces the virulence factors. Upon sensing, autophosphorylation of the histidine

kinase, VirA occurs at the conserved histidine residue His474 [337-339]. This leads to the transfer of the phosphoryl group to conserved aspartate residue, Asp52 of the cognate response regulator VirG and is known for its unique stability in the phosphorylated form [340]. This transcription factor VirG, controls the expression of the protein virB [341].

6.1.4 Role of synthetic biology in engineering two-component signaling systems

The broad engineering capability attributed to synthetic biology is to be able to modify and tune entire systems and give them novel functions. Systems biology plays a crucial role by developing the required high throughput technologies such as ultrasequencing, DNA microarrays, automated microscopy, mass spectrometry and computation [342]. Synthetic Biology studies focused on studying positive and negative feedback loops, noise, oscillations and the system robustness initially [343-346,136,137]. Subsequent studies focused on designing gene circuits coupled with metabolism and gene expression, quorum sensing pathways, synthesis of new chemical compounds such as terpenoids, study network evolution and spatial patterning [347-352].

Taking cue from bacterial systems, synthetic circuits were designed in eukaryotic systems which have shown tremendous development in terms of their complexity [353]. This includes the use of repressor proteins and RNA interference in the modular design of inducible gene silencing alongside the first synthetic tunable

mammalian oscillator in human embryonic kidney (HEK293) cells and Chinese Hamster Ovary (CHO) cells [354,355].

6.1.5 Design considerations for two-component signaling systems

Signaling pathways transmit the signal and react very spontaneously ranging from milliseconds to a few minutes [356]. The main considerations while designing the system are the general system properties and its components [342]. Pathway engineering involves protein localization, assembly, activation-deactivation, competition, diffusion, active transport, degradation, modularity, feedback loops, specificity and crosstalk [357-361]. These characteristics are to be taken into consideration while designing or modifying systems. The overall system behavior depends of each of the parameters considered and altered.

6.1.6 Invasin – Bacterial protein that binds to mammalian cells

Numerous bacterial pathogens enter nonphagocytic cells cultured *in vitro* [362]. Enteropathogenic *Yersinia* enters host cells to obtain access to sub-epithelial regions which is part of the systemic disease leading to infection of lymph nodes [363]. Invasin protein derived from *Yersinia* is enough to impart to *E. coli* the ability to invade host cells via the $\beta 1$ -integrins expressed on the mammalian cells [364]. The C-terminus 192 amino acids is the shortest domain of invasin which imparts to this protein the ability to bind to $\beta 1$ -integrins [365]. The Asp911 residue is essential for binding to $\beta 1$ -integrins and this residue is similar to the asp residue in the RGD motif of fibronectin which allows binding of fibronectin to $\alpha 5\beta 1$ integrins [366]. It was later shown that these 192 amino acids domain not only binds to $\beta 1$ -integrins,

but is also sufficient for the internalization of the bacterium into the mammalian cells [367]. In comparison to fibronectin, invasin is more efficient in the uptake of bacteria by mammalian cells even when the number of $\beta 1$ -integrins is lower on the mammalian cells as the affinity between invasin and $\beta 1$ -integrins is much higher [368].

6.1.7 Objectives of this chapter

This work aims to develop an engineered *E. coli* that harbors synthetic circuits capable of responding to colorectal cancer cells. This response circuit would consist of a sensing domain which is a membrane protein capable of detecting the external signal (here $\beta 1$ integrins). This chapter focuses on the construction of this sensing histidine kinase in the bacterial two-component signaling system which is engineered to possess a $\beta 1$ -integrin binding domain of invasin (Inv) attached to cytoplasmic domains of histidine kinases EnvZ and VirA. The complete sensing circuit can be found in **Figure 6.2**.

Figure 6.2: InvVirA-VirG (above) and InvEnvZ-OmpR (below) engineered two-component circuits to bind to $\beta 1$ integrins. InvVirA and InvEnvZ are the histidine kinases, VirG and OmpR are the response regulators, and RFP is the signal output.

In this chapter, chimeric membrane proteins that can bind to β 1-integrins on colorectal cancer cells are constructed. The binding of the engineered bacterium is assessed with colorectal cancer followed by investigation on the time course of internalization of the engineered bacterium into colorectal cancer.

6.2 Materials and Methods

6.2.1 *E. coli* strains and plasmids

E. coli TOP10 (Invitrogen) strains for the construction, maintenance and expression of plasmids. *E. coli* DH10B cells were used when *E. coli* TOP10 failed to produce colonies after transformation. Plasmids pBbe8k with Kanamycin resistance (<https://www.addgene.org/35270/>) and pBbe8a with ampicillin resistance (<https://www.addgene.org/35268/>) were used in the cloning [369].

6.2.2 Parts used in the plasmids

In all the constructs, the ribosome binding site (RBS) used was B0034 (http://parts.igem.org/Part:BBa_B0034). Promoter J23101 was used for constitutive expression of proteins (http://parts.igem.org/Part:BBa_J23101). Part B0015 which is a combination of parts B0010 and B0012 was used as the transcriptional terminator (http://parts.igem.org/Part:BBa_B0015). Inducible expression of the proteins is achieved by pBAD promoter present in pBbe8k and pBbe8a.

6.2.3 Plasmid construction

Standard restriction digestion and ligation protocols were used to clone two fragments together which follow the RFC 21 standard biobrick assembly method [370]. This standard uses the enzymes EcoRI and BglII as the prefix and BamHI and XhoI as the suffix. DNA assembly methods have been described in Appendix IV. Gibson assembly method was also used to assemble various parts. Details of each construct is mentioned below.

Construct 1: InvEnvZ fusion protein consists of 192 amino acids of the β 1 integrin binding domain of invasin fused to the cytoplasmic domain of EnvZ. InvEnvZ fusion protein in pBbe8k constructed using biobrick cloning. InvEnvZ fusion gene along with B0034 RBS was ordered from Integrated DNA Technologies, Singapore and cloned between sites BglII and XhoI in pBbe8k. This fusion gene was then assembled into pBbe8k plasmid containing constitutive promoter J23101 and RBS B0034 by Gibson assembly resulting in InvEnvZ under J23101 constitutive promoter. Details of primers and templates can be found in Appendix IV.

Construct 2: InvVirA fusion protein in pBbe8k constructed using biobrick cloning. InvVirA fusion gene along with B0034 RBS was ordered from Integrated DNA Technologies, Singapore and cloned between sites BglII and XhoI in pBbe8k. This fusion gene was then assembled into pBbe8k plasmid containing constitutive promoter J23101 and RBS B0034 by Gibson assembly resulting in InvVirA under J23101 constitutive promoter. Details of primers and templates can be found in Appendix IV.

Construct 3: Invasin protein in pBbe8k constructed using biobrick cloning. Invasin gene along with B0034 RBS was ordered from Integrated DNA Technologies, Singapore and cloned between sites BglII and XhoI in pBbe8k. This gene was then assembled into pBbe8k plasmid containing constitutive promoter J23101 and RBS B0034 by Gibson assembly resulting in Invasin under J23101 constitutive promoter. Details of primers and templates can be found in Appendix IV.

Construct 4: RFP was attached to the C-terminus of InvEnvZ gene by Gibson assembly. RFP was amplified from pBbe8k and construct 1 was amplified in 2 parts. All three parts were assembled by Gibson assembly generating RFP fusion protein in the cytoplasmic part of InvEnvZ. Details of primers and templates can be found in Appendix IV.

6.2.4 Scanning electron microscopy on co-cultured cells

Colorectal cancer cells HCT116 and SW480 were seeded as 125,000 cells per well in 24 well cell culture plates with Thermanox tissue culture treated coverslips. Cells were allowed to adhere and spread for 24 hours. Overnight cultures of constitutively expressing InvEnvZ and InvVirA *E. coli* TOP10 and control *E. coli* TOP10 were prepared. Bacteria were resuspended in McCoy's 5a and RPMI medium to make MOI 50 ratio to cancer cells. Wells were washed with PBS and medium containing bacteria was added to the wells and incubated at 37 °C, 5% CO₂ for 2 hours. Wells were then washed 4 times with DPBS to remove unbound bacteria. 1 ml of primary fixative 2.5% glutaraldehyde in PB was added and the plates were kept at 4 °C overnight. Wells were washed with 0.1M PB thrice. Secondary fixative 1% osmium tetroxide was added to the wells and incubated in the fume hood for 1 hours. Wells were washed with PB thrice. Dehydration of the sample was performed with a graded ethanol series wash consisting of 40%, 60%, 80%, 90%, 95%, 100%, 100% ethanol. 50% HMDS in ethanol was added and removed after 5 minutes followed by 75% HMDS in ethanol and 100% HMDS. 100 µl of HMDS was added to the wells and left in the fume hood to dry overnight following which images were

captured in Jeol JSM 6700F FESEM by sticking the coverslip to double sided carbon tape on SEM stub and sputter coated with platinum.

6.2.5 Gentamicin protection assay

Gentamicin protection assay was carried out by using a modified protocol [371]. Overnight cultures (5 ml) of constitutively expressed InvEnvZ and InvVirA in *E. coli* TOP10 were prepared along with *E. coli* TOP10 as the control. On the same day 175,000 cells, each of HCT116 and SW480 were seeded in 24 well plates with 1ml of McCoy's 5A and RPMI media respectively and allowed to adhere and grow for 24 hours at 37 °C, 5% CO₂, when the confluency reaches approximately 75%. Following day, bacteria was diluted in both McCoy's 5A media and RPMI media to a Multiplicity of index (MOI, ratio of bacteria to mammalian cells) (50, 500 and 1000) in 500 µl by measuring the optical density at 600 nm (Optical density of 1 is equivalent to 10⁹ *E. coli* cells per ml). The medium in the plates was replaced with the medium containing bacteria and the plates were incubated at 37°C, 5 % CO₂ for 2 hours. The medium in the plates was then removed and wells were washed with sterile DPBS and fresh medium containing 100 µg/ml of gentamicin was added and the plates were incubated at 37°C, 5% CO₂ for 1 hour. Medium was then removed, and the wells were washed 4 times with sterile DPBS to remove any residual gentamicin. Cells were then lysed with 500 µl of DPS containing 0.2% Triton X-100. Solution was diluted 100 times and 50 µl was plated on Streptomycin (100 µg/ml) – Kanamycin (100 µg/ml) LB agar plates and all the plates were incubated at 37°C overnight and colonies on the plates were counted the following day.

6.2.6 Confocal microscopy

Confluent monolayers of HCT116 and SW480 were made on Ibidi 8 well chambers by seeding 150,000 cells per well and allowing them to adhere and spread for 24 hours. Bacterial samples of InvEnvZ-RFP (InvEnvZ fused with RFP at the C-terminus), Invasin+RFP (pBbE8k/Invasin and pBbE8a/RFP co-transformed into *E. coli* TOP10) and RFP (pBbE8a/RFP) were prepared by diluting 1/100 overnight cultures in fresh LB containing required antibiotics and induced with 0.5% arabinose (to express RFP in pBbE8a) at OD~0.5 and continued to grow for 8 hours. HCT116 and SW480 were incubated with anti- β 1-integrin antibody in McCoy's 5A and RPMI medium respectively for 2 hours while control wells were maintained only in respective media. Required number of bacteria to reach MOI 50 were diluted in McCoy's 5A and RPMI medium and 200 μ l of different bacteria containing medium was added to HCT116 and SW480. Plates were incubated at 37°C, 5% CO₂ for different time periods (2 hours, 4 hours, 6 hours and 8 hours). Cells were washed 4 times with DPBS to remove unbound bacteria and cells were fixed with 4% PFA for 15 mins. Wells were then washed 3 times with DPBS to remove residual PFA and wells that were previously not blocked with anti- β 1-integrins were now treated with 250 μ l of Superblock buffer (Thermo Fisher Scientific) and incubated on a rocker for 2 hours at RT. These well were then washed thrice with PBS containing 0.05% Tween-20 were treated with 200 μ l of 0.1% Triton X-100 in PBS for 10 minutes and then washed thrice with PBS. 50 μ l of anti- β 1-integrin antibody was added to these wells and incubated at RT for 1 hour on a rocker. Wells were washed with PBS thrice. Further steps were carried out on all the wells. 100 μ l of NucBlue

(ThermoFisher Scientific) solution (2 drops in 1 ml PBS) was added to all the wells and incubated for 15 minutes to stain the nucleus. Wells were washed twice with PBS and all the PBS was removed from the wells and the chamber was detached. 50 μ l of mowiol mounting medium was added on the glass slide and mounted with a cover slip and left in the fume hood overnight. The mounted slides were stored at 4°C and images were captured using Zeiss LSM 800 confocal microscope.

6.3 Results and Discussions

This section discusses the binding of chimeric membrane proteins to β 1-integrins on colorectal cancer cells. Binding of engineered bacterium to HCT116 and SW480 colorectal cancer cells is studied by SEM and internalization of the bacterium is enumerated by gentamicin protection assay. To study the time course of internalization, engineered bacteria were incubated with HCT116 and SW480 for varied time points and internalization was monitored using confocal microscopy.

6.3.1 Plasmid construction

InvEnvZ (construct 1) and InvVirA construct 2), being chimeric membrane proteins, were difficult to clone into the pBbE8a plasmid. Many variations in the Gibson assembly protocol were tried by changing the ratio of fragments, using a longer assembly time but neither helped in obtaining the desired plasmid. It is hypothesized that the membrane protein being expressed constitutively in the bacterial cell, adds pressure in the transformation step, chopping the protein part out from the plasmid resulting in truncated plasmids. This problem was partially circumvented by tweaking the transformation step by incubating the transformed plates at room temperature for 2 days instead of at 37°C for 16 hours. The plasmid maps of the successful clones along with the assembly primers and templates are presented in Appendix IV.

Here, the successful clones include, constitutive expression of the chimeric membrane proteins (InvVirA and InvEnvZ). Invasin protein was also cloned under constitutive promoter J23101 (construct 3). RFP fusion to C-terminus of InvEnvZ

where RFP is expressed in the cytoplasmic part of InvEnvZ (construct 4) was also constructed. *E. coli* TOP10 has been used as the host in this part of the study because we aim to make low levels of the constitutively expressed chimeric protein. In the future, we aim to produce these proteins in probiotic *E. coli* Nissle strain which may have lower protein expression than TOP10 and may require genome integration.

6.3.2 Scanning electron microscopic analysis of bacterial adhesion to colorectal cancer cells

To investigate the binding of the engineered bacteria containing the chimeric proteins to mammalian cells, bacteria were incubated for 2 hours with confluent monolayers of colorectal cancer cells HCT116 and SW480. After incubation, the cells were washed several times to remove the unbound bacteria and samples were prepared for scanning electron microscopy. Binding of the chimeric proteins (InvEnvZ and InvVirA) expressing bacteria was observed in both cells lines HCT116 (**Figure 6.3**) and SW480 (**Figure 6.4**).

Pathogenic bacteria *Yersinia pseudotuberculosis* express invasins that are 986 amino acids long on the bacterial membrane that are known to bind to β 1-integrins that are expressed on the mammalian cells and the bacterium is internalized by zipper mechanism [372-374]. Later the binding domain of the invasin protein was identified to be C-terminus 192 amino acids. [365]. Our results from the scanning electron microscopic analysis show that the bacteria expressing invasin binding domain do bind to colorectal cancer cells within 2 hours of co-incubation and is in agreement with the previously published literature [372-374]. Control study with

bacteria not expressing any of the chimeric proteins did not show binding to the mammalian cells in 2 hours of incubation. Further experiments were conducted to identify whether the c-terminus binding domain of invasin leads to internalization of the bacterium into colorectal cancer cells.

Figure 6.3: Engineered *E. coli* TOP10 expressing InvEnvZ and InvVirA at an MOI 50 incubated for 2 hours adhere to colorectal cancer cell line HCT116. Control experiment with non-engineered *E. coli* TOP10 cells show no binding. Scale bar for images on the left represents 10 μm and zoomed in version with scale bar representing 1 μm is presented on the right.

Figure 6.4: Engineered *E. coli* TOP10 strain expressing InvEnvZ and InvVirA at an MOI 50 incubated for 2 hours adhere to colorectal cancer cell line SW480. Control experiment with non-engineered *E. coli* TOP10 cells show no binding. Scale bar for images on the left represents 10 μm and zoomed in version with scale bar representing 1 μm is presented on the right.

6.3.3 Internalization of engineered bacteria

Scanning electron microscopic analysis showed that the bacteria expressing a domain of invasin is able to bind to colorectal cancer cells. Further studies were conducted to ascertain whether the binding lead to internalization of the bacteria. Gentamicin protection assay which quantifies the number of bacteria internalized into mammalian cells was conducted to enumerate the internalization.

This assay involves the co-incubation of bacterial cells with colorectal cancer cells for a desired amount of time for the invasion to occur. After the invasion, the mammalian cells are washed and treated with gentamicin to kill any bacteria that is either unbound or bound to the surface of the mammalian cells. After removing the gentamicin, mammalian cells are lysed and the internalized bacteria is enumerated as colony forming units.

This assay was conducted with the cells expressing chimeric proteins (InvEnvZ and InvVirA) and control bacteria *E. coli* TOP10. Invasion assay was carried out for 2 hours and the assay was conducted with varying ratios of bacteria (MOI 50, MOI 500 and MOI 1000) to cancer cells (HCT116 and SW480).

As can be seen from **Figure 6.5**, around 700,000 of the engineered bacteria (InvEnvZ and InvVirA) were internalized in the colorectal cancer cells HCT116 and SW480. Increase in the ratio of bacteria to the cancer cells (50, 500 and 1000) did not significantly increase the number of bacteria internalized and this relationship does not seem to be proportional. Around 40,000 cells of *E. coli* TOP10 were internalized by the cancer cells which is significantly lower than the engineered

bacteria clearly indicating that the invasin domain on the bacterial cells has aided in the internalization of the bacterial cells by the colorectal cancer cells.

Figure 6.5: Bacterial internalization study by gentamicin protection assay performed at different MOI ratios and co incubated with HCT116 and SW480 for 2 hours.

Since there is no significant increase in the number of bacteria internalized upon increasing the Multiplicity of Index (MOI) which is the ratio of bacteria to mammalian cells added, percentage of bacterial internalization was calculated (**Figure 6.6**). This was calculated by correlating the number of colony forming units obtained to the original number of bacteria added during co-incubation. It was observed that at MOI 50, 6.5% of InvEnvZ bacteria and 9% of InvVirA bacteria was internalized by HCT116. 8.5% of InvEnvZ bacteria and 6.5% of InvVirA bacteria was internalized by SW480. However, only around 1% and 0.5% of the engineered

bacteria were internalized with MOI500 and MOI1000 bacteria respectively. Since the efficiency of internalization is the maximum for MOI50, this ratio was chosen for all the experiments.

Other studies have shown that invasin expressing *E. coli* cells were internalized by a range of mammalian cells such as HeLa cells with 8% internalization, U2OS (osteosarcoma) cells with 2.9% internalization and HepG2 (hepatocarcinoma) cells with 0.2% internalization [145]. The differences in the amount of internalization by different cell lines depends on the exposed β 1-integrins on the cell surface. Also, the invasion is more efficient on cells that actively express β 1-integrins as in the case of growing edges of epithelial sheets [375].

Figure 6.6: Percentage of bacterial internalization when bacteria at different MOIs are co-incubated with HCT116 and SW480 for 2 hours.

6.3.4 Time course of bacterial entry into cancer cells through β 1-integrins

From the previous experiments, it is ascertained that the chimeric proteins have facilitated the entry of bacteria into colorectal cancer cells. The invasin protein is shown to bind to β 1-integrins of mammalian cells leading to their uptake [364]. To identify whether the uptake of bacterial cells harboring the chimeric proteins is in fact driven by binding to β 1-integrins of the colorectal cancer cells, we have conducted experiments with co incubation of chimeric protein expressing bacteria with colorectal cancer cells while also having controls where the integrins are blocked by using anti β 1-integrin antibody prior to introducing the bacteria. Also, we have conducted the uptake experiments at different time points to identify the time duration of selectivity conferred to the bacterial cells by virtue of the chimeric proteins.

Red fluorescent protein (RFP) was fused to the C-terminus of the InvEnvZ generating the C-terminus fusion protein InvEnvZ-RFP (construct 4) where RFP would be expressed in the cytoplasm part of the chimeric protein. We have unfortunately not been able to generate RFP fusion protein with InvVirA as the transformation always yielded in truncated plasmids. Hence, we have proceeded with the uptake experiments with InvEnvZ-RFP fusion protein alone using confocal microscopy.

Colorectal cancer (HCT116 and SW480) confluent monolayers were formed and the cells were treated with anti- β 1-integrin antibody fused with Alexa Fluor 488. This ensured that the β 1-integrins on the cancer cells were blocked in the controls and were unavailable for binding to invasin protein on bacterial cells, while the cells

without antibody treatment had their integrins available for binding to invasin. Bacteria harboring InvEnvZ-RFP in pBbE8k, negative control plasmid pBbE8k containing RFP alone and positive control invasin full protein expressed along with RFP was induced with 0.5% arabinose for the expression of the respective proteins. These induced bacteria were co-incubated with colorectal cancer cells with blocked/unblocked integrins for different time periods (2hours, 4 hours, 6 hours and 8 hours) post which, the cells were washed and fixed. The control wells where the integrins were not blocked prior to the introduction of bacteria, were stained with anti- β 1-integrin antibody after fixing the cells. DAPI staining was done on all the samples to stain the nucleus.

Confocal micrographs (20x magnification) were taken for various times of co-incubation with and without the integrins blocked prior to the introduction of bacteria. As can be seen from **Figure 6.10**, InvEnvZ-RFP and invasin+RFP were internalized into the cancer cells as early as 2 hours only when the integrins were not blocked before introducing the bacteria. This selective invasion was observed at 4 hours as well, in both InvEnvZ-RFP and the positive control invasin+RFP. This trend was the same both in HCT116 and SW480. The negative control RFP did not show invasion during the first 4 hours however, after 4 hours, bacteria were seen to be localized in the cancer cells and adhered to the cancer cells. This was also the case with blocked β 1-integrin cancer cells. 6 hours and 8 hours co-incubation of bacteria with colorectal cancer cell lead to internalization of bacteria despite the integrins being blocked.

This experiment concludes that the bacteria have conferred selectivity for invasion into colorectal cancer cells, through the β 1-integrins expressed on the cancer cells and this selectivity was clearly visible for 4 hours. It is a possibility that the integrins might not have been completely blocked and hence the entry of the bacteria into the mammalian cells could have been delayed in samples with blocked integrins leading to delayed internalization after 6 hours. However, negative control cells which do not have invasin have also entered the cancer cells after 4 hours. Hence, another route of entry into the cancer cells after 4 hours is also a possibility.

Figure 6.7: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with HCT116 for (a) 2 hours, (b) 4 hours, Nucleus is stained blue, integrins are stained green and bacteria is red.

Figure 6.8: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with HCT116 for (c) 6 hours, (d) 8 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.

Figure 6.9: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with SW480 for (e) 2 hours, (f) 4 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.

Figure 6.10: Confocal micrographs of invasion of bacteria (RFP, Invasin+RFP, InvEnvZ-RFP) with SW480 for (g) 6 hours, and (h) 8 hours. Nucleus is stained blue, integrins are stained green and bacteria is red.

6.4 Conclusions

The chimeric proteins InvEnvZ and InvVirA were successfully cloned and expressed in *E. coli*. Binding of these engineered bacteria was shown to HCT116 and SW480 through scanning electron microscopy. The internalization of these engineered bacteria by HCT116 and SW480 was enumerated at different ratios of co-incubation and around 8% of the bacteria was seen to be internalized by the cancer cells at an MOI50. This study also shows that the 192 amino acids in Invasin protein are sufficient for binding to colorectal cancer cells.

The time course of the bacterial internalization and the internalization route was investigated and selective invasion was observed up to 4 hours via β 1-integrin mediated uptake in InvEnvZ-RFP. Beyond 4 hours, cells with and without invasin were able to enter colorectal cancer cells and the route of their entry is not yet clear.

In this chapter, the sensing part of the synthetic circuit was successfully completed and characterized for its binding ability and selectivity. Further studies would include the completion of the synthetic circuit and testing its ability to respond to colorectal cancer cells.

Chapter 7: Concluding Remarks and Future Directions

In this thesis, various aspects of colorectal cancer management were addressed where the focus was laid on the development of tools for diagnostic, therapeutic and specific targeting of colorectal cancer. In the first work, magnetic bacterium was engineered to be able to over load iron and make iron nanoparticles *in vivo* which could potentially be used for MRI diagnosis of colorectal cancer. In the second work, thermophilic ferritin nanocages were encapsulated with photosensitizer molecule for therapeutic applications of colorectal cancer. In the final chapter, *E. coli* able to bind to $\beta 1$ integrin of colorectal cancer was developed and this bacterium was shown to be internalized by colorectal cancer cells. Some perspectives on the future development of each project are elucidated in more details in respective sections.

7.1.1 Oral diagnostics for detection of colorectal cancer

By employing a synthetic biology approach, various functionalities have been imparted to commensal bacterium *E. coli*. By increasing the iron influx pump and blocking the efflux pump, iron nanoparticles were synthesized in the cytoplasm of *E. coli* which would act as MRI contrast agents in cancer diagnostics. The magnetic properties of the bacterium were improved by engineering magnetite forming peptide in the nano-carrier, AfFtn. This engineered approach aided in the construction of *E. coli* that could be ingested for its accumulation in GI tract and eventually help in diagnosis of tumors.

Understanding of the magnetic properties of the material is important to enable tailoring to specific needs. Hence, it is imperative to understand the exact forms of iron that is mineralized in AfFtn-m6A [376]. Relaxivities of these magnetic bacteria needs to be obtained *in vitro* by making agarose phantoms of serially diluted bacterial concentrations and measuring their contrast enhancement [130]. The next course of the study would include testing the effectiveness of these MRI contrast generating microbes in mice models.

7.1.2 AfFtn as a drug nano-carrier

Protein nanocages derived from bacteria, were loaded with photosensitizers to act as cytotoxic agents in photodynamic therapy. This was achieved by employing a cost-effective home LED lighting which would reduce the costs of the photosensitization setup. This approach has paved way for fast, cheap and effective tumor therapy. Ionic strength mediated and ferrous ion mediated encapsulation of photosensitizers in ferritin nanocages was demonstrated in this study which paves way to the encapsulation of various other small molecules in the ferritin nanocages. Near infra- red dyes could be encapsulated for use in photodynamic therapy of melanoma.

Since the route of cell signaling responsible for the induction of cell death defines the kind of cytotoxic stress (expression of proteins, and regulation of angiogenesis factors) levied on the tumors, it is important to study how AfFtn is internalized into colorectal cancer cell. Previous reports on internalization shows that the path of internalization is related to route of cell death [240]. Our results suggest

that the internalized cages are present in the endosomes and this opens up new arenas for designing cages with endosomal escape properties [377]. Nanoscale materials are endocytosed into mammalian cells by various pathways and hence to gain better understanding of the endocytosis mechanism, each pathway could be blocked with specific inhibitors to elucidate the exact internalization process [226].

Researchers today have been developing novel nano-sized drug delivery platforms with various properties but little focus has been put towards understanding their bio-distribution and residence time in the body [378,379]. These studies are extremely important to assess the suitability of the vehicle in humans for drug delivery. Bio-distribution studies of the drug delivery vehicles would also reflect on the suitability for specific applications based on their retention in different organs.

Cytotoxicity of various drug molecules are tested for their efficacy on 2D cell monolayers. Although, this shows to some extent the effectiveness of the drug, it might not always reflect in the real case scenario [380]. To be able to screen for drugs better, 3D models need to be employed which would be a better screening platform [381,382]. Intestinal organoid models are also available which maintain the vital aspects of intestinal physiology where high throughput therapeutic screening can be performed [383,384]. These 3D models could also be employed to test for the efficacy of photosensitizers in PDT. Drug loaded Afftn nanocages could be assessed for their killing efficiency on 3D models of colorectal cancer cell lines. These platforms could be used for the assessment of dosage and frequency of treatment.

7.1.3 Bacterial engineering to respond to colorectal cancer cells

The engineering of the bacteria to bind and enter colorectal cancer was completed in this study. The chimeric sensing protein was successfully tested for its binding ability to colorectal cancer cells. Its functionality remains to be established. The chimeric protein should be able to respond and phosphorylate upon binding to colorectal cancer (or $\beta 1$ integrins). Functional assays to establish the phosphorylation of the membrane protein are required to ascertain the binding ability and affinity to $\beta 1$ integrins. Identification of the bottlenecks in this approach and re-engineering of the circuits to effectively respond to colorectal cancer remains to be explored.

Once, the functionality of the circuit is established, future investigations would involve testing the effectiveness of this engineered bacterium in mice colorectal tumor models. Mice need to be fed the engineered bacteria, capable of colonization and binding to the colorectal tumors. This specificity could be detected by the reporter gene RFP expression in the colorectal tumors.

The ultimate goal of the project is the construction of a dual functional bacterium capable of specific binding to colorectal cancer and simultaneously possess magnetic nanoparticles for the development of a diagnostic tool in the MRI of colorectal region. Each component of this diagnostic tool (specificity and magnetic nanoparticles) has already been established individually. Further work needs to be accomplished in combining these two systems into a single bacterium to assess its efficacy in MRI diagnosis of colorectal tumors.

The immediate experiments to achieve the mentioned goal, would be to first ascertain the contrast enhancement ability of the iron nanoparticle synthesizing

bacterium. The subsequent experiment would include testing the synthetic circuit. The transfer of the external stimuli ($\beta 1$ integrin binding) is crucial to obtain response from the engineered signaling circuit.

References

- [1] Andrianantoandro, E., Basu, S., Karig, D. K., Weiss, R., Synthetic biology: new engineering rules for an emerging discipline. *Mol Syst Biol* 2006, 2, 2006 0028.
- [2] Khalil, A. S., Collins, J. J., Synthetic biology: applications come of age. *Nat Rev Genet* 2010, 11, 367-379.
- [3] Levskaya, A., Chevalier, A. A., Tabor, J. J., Simpson, Z. B., *et al.*, Synthetic biology: engineering *Escherichia coli* to see light. *Nature* 2005, 438, 441-442.
- [4] Tabor, J. J., Salis, H. M., Simpson, Z. B., Chevalier, A. A., *et al.*, A synthetic genetic edge detection program. *Cell* 2009, 137, 1272-1281.
- [5] Lee, S., Mattanovich, D., Villaverde, A., Systems metabolic engineering, industrial biotechnology and microbial cell factories. *Microbial Cell Factories* 2012, 11.
- [6] Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., Keasling, J. D., Microbial engineering for the production of advanced biofuels. *Nature* 2012, 488, 320-328.
- [7] World Health Organization - Cancer Keyfacts <http://www.who.int/mediacentre/factsheets/fs297/en/>.
- [8] American Cancer Society - Colorectal Cancer <https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html>.
- [9] Forbes, N. S., Engineering the perfect (bacterial) cancer therapy. *Nat Rev Cancer* 2010, 10, 785-794.
- [10] Saunders, T. H., New techniques for imaging colorectal cancer: the use of MRI, PET and radioimmunoscintigraphy for primary staging and follow-up. *British Medical Bulletin* 2002, 64, 81-99.
- [11] Lu, J., Ma, S., Sun, J., Xia, C., *et al.*, Manganese ferrite nanoparticle micellar nanocomposites as MRI contrast agent for liver imaging. *Biomaterials* 2009, 30, 2919-2928.
- [12] Khawaja, A. Z., Cassidy, D. B., Al Shakarchi, J., McGrogan, D. G., *et al.*, Revisiting the risks of MRI with Gadolinium based contrast agents-review of literature and guidelines. *Insights Imaging* 2015, 6, 553-558.
- [13] Mornet, S. p., Vasseur, S. b., Grasset, F., Duguet, E., Magnetic nanoparticle design for medical diagnosis and therapy. *Journal of Materials Chemistry* 2004, 14, 2161.
- [14] Fossheim, S. L., Fahlvik, A. K., Klaveness, J., Muller, R. N., Paramagnetic liposomes as MRI contrast agents: influence of liposomal physicochemical properties on the in vitro relaxivity. *Magnetic Resonance Imaging* 1999, 17, 83-89.
- [15] Yang, F., Li, Y., Chen, Z., Zhang, Y., *et al.*, Superparamagnetic iron oxide nanoparticle-embedded encapsulated microbubbles as dual contrast agents of magnetic resonance and ultrasound imaging. *Biomaterials* 2009, 30, 3882-3890.
- [16] Mulder, W. J., Strijkers, G. J., van Tilborg, G. A., Griffioen, A. W., Nicolay, K., Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. *NMR Biomed* 2006, 19, 142-164.
- [17] Archibald, F., *Lactobacillus plantarum*, an organism not requiring iron. *FEMS Microbiology Letters* 1983, 19, 29-32.
- [18] Posey, J. E., Lack of a Role for Iron in the Lyme Disease Pathogen. *Science* 2000, 288, 1651-1653.

- [19] Shi, J., Kantoff, P. W., Wooster, R., Farokhzad, O. C., Cancer nanomedicine: progress, challenges and opportunities. *Nat Rev Cancer* 2017, 17, 20-37.
- [20] Schroeder, A., Heller, D. A., Winslow, M. M., Dahlman, J. E., *et al.*, Treating metastatic cancer with nanotechnology. *Nat Rev Cancer* 2011, 12, 39-50.
- [21] Toso, J. F., Gill, V. J., Hwu, P., Marincola, F. M., *et al.*, Phase I study of the intravenous administration of attenuated *Salmonella typhimurium* to patients with metastatic melanoma. *J Clin Oncol* 2002, 20, 142-152.
- [22] Danino, T., Prindle, A., Kwong, G. A., Skalak, M., *et al.*, Programmable probiotics for detection of cancer in urine. *Sci Transl Med* 2015, 7, 289ra284.
- [23] Pasula, R. R., Lim, S., Engineering nanoparticle synthesis using microbial factories. *Engineering Biology* 2017, 1, 12-17.
- [24] Salata, O., Applications of nanoparticles in biology and medicine. *Journal of Nanobiotechnology* 2004, 2.
- [25] Haruta, M., When gold is not noble: catalysis by nanoparticles. *Chem Rec* 2003, 3, 75-87.
- [26] Luangpipat, T., Beattie, I. R., Chisti, Y., Haverkamp, R. G., Gold nanoparticles produced in a microalga. *Journal of Nanoparticle Research* 2011, 13, 6439-6445.
- [27] Daniel, M.-C., Astruc, D., Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chemical Reviews* 2004, 104, 293-346.
- [28] Mandal, D., Bolander, M. E., Mukhopadhyay, D., Sarkar, G., Mukherjee, P., The use of microorganisms for the formation of metal nanoparticles and their applications. *Applied microbiology and biotechnology* 2006, 69, 485-492.
- [29] Campisi, S., Schiavoni, M., Chan-Thaw, C., Villa, A., Untangling the Role of the Capping Agent in Nanocatalysis: Recent Advances and Perspectives. *Catalysts* 2016, 6, 185.
- [30] Campisi, S., Chan-Thaw, C. E., Wang, D., Villa, A., Prati, L., Metal nanoparticles on carbon based supports: The effect of the protective agent removal. *Catalysis Today* 2016, 278, 91-96.
- [31] Lopez-Sanchez, J. A., Dimitratos, N., Hammond, C., Brett, G. L., *et al.*, Facile removal of stabilizer-ligands from supported gold nanoparticles. *Nat Chem* 2011, 3, 551-556.
- [32] Thakkar, K. N., Mhatre, S. S., Parikh, R. Y., Biological synthesis of metallic nanoparticles. *Nanomedicine : nanotechnology, biology, and medicine* 2010, 6, 257-262.
- [33] Zhang, X., Yan, S., Tyagi, R. D., Surampalli, R. Y., Synthesis of nanoparticles by microorganisms and their application in enhancing microbiological reaction rates. *Chemosphere* 2011, 82, 489-494.
- [34] Lobo, M. B., Vasconcelos, J. V., Resistance of *Mycobacterium tuberculosis* to oligodynamic action of silver. *Revista brasileira de tuberculose e doencas toracicas* 1950, 18, 647-654.
- [35] Becking, L. G. M. B., Kaplan, I. R., Moore, D., Limits of the Natural Environment in Terms of pH and Oxidation-Reduction Potentials. *The Journal of Geology* 1960, 68, 243-284.
- [36] Blakemore, R., Magnetotactic bacteria. *Science* 1975, 190, 377.

- [37] Charley, R. C., Bull, A. T., Bioaccumulation of silver by a multispecies community of bacteria. *Archives of Microbiology* 1979, 123, 239-244.
- [38] Bazyliniski, D. A., Frankel, R. B., Jannasch, H. W., Anaerobic magnetite production by a marine, magnetotactic bacterium. *Nature* 1988, 334, 518-519.
- [39] Aiking, H., Kok, K., van Heerikhuizen, H., van 't Riet, J., Adaptation to Cadmium by *Klebsiella aerogenes* Growing in Continuous Culture Proceeds Mainly via Formation of Cadmium Sulfide. *Applied and Environmental Microbiology* 1982, 44, 938-944.
- [40] Mann, S., Mineralization in biological systems, in: Connett, P. H., Follmann, H., Lammers, M., Mann, S., *et al.* (Eds.), *Inorganic Elements in Biochemistry*, Springer Berlin Heidelberg, Berlin, Heidelberg 1983, pp. 125-174.
- [41] Kang, S. H., Bozhilov, K. N., Myung, N. V., Mulchandani, A., Chen, W., Microbial synthesis of CdS nanocrystals in genetically engineered *E. coli*. *Angewandte Chemie* 2008, 47, 5186-5189.
- [42] Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., *et al.*, Bioreduction of AuCl₄⁻ Ions by the Fungus, *Verticillium* sp. and Surface Trapping of the Gold Nanoparticles Formed D.M. and S.S. thank the Council of Scientific and Industrial Research (CSIR), Government of India, for financial assistance. *Angewandte Chemie International Edition* 2001, 40, 3585.
- [43] Lengke, M. F., Fleet, M. E., Southam, G., Morphology of gold nanoparticles synthesized by filamentous cyanobacteria from gold(I)-thiosulfate and gold(III)-chloride complexes. *Langmuir* 2006, 22, 2780-2787.
- [44] Liu, J., Zhang, X., Yu, M., Li, S., Zhang, J., Photoinduced silver nanoparticles/nanorings on plasmid DNA scaffolds. *Small* 2012, 8, 310-316.
- [45] Watson, J. D., Crick, F. H. C., Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. *Nature* 1953, 171, 737-738.
- [46] Cohen, S. N., Chang, A. C. Y., Boyer, H. W., Helling, R. B., Construction of Biologically Functional Bacterial Plasmids In Vitro. *Proceedings of the National Academy of Sciences of the United States of America* 1973, 70, 3240-3244.
- [47] Itakura, K., Hirose, T., Crea, R., Riggs, A., *et al.*, Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* 1977, 198, 1056-1063.
- [48] Goeddel, D. V., Heyneker, H. L., Hozumi, T., Arentzen, R., *et al.*, Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature* 1979, 281, 544-548.
- [49] Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., *et al.*, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988, 239, 487-491.
- [50] Hitzeman, R. A., Hagie, F. E., Levine, H. L., Goeddel, D. V., *et al.*, Expression of a human gene for interferon in yeast. *Nature* 1981, 293, 717-722.
- [51] Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., *et al.*, Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 2006, 440, 940-943.
- [52] Mi, C., Wang, Y., Zhang, J., Huang, H., *et al.*, Biosynthesis and characterization of CdS quantum dots in genetically engineered *Escherichia coli*. *Journal of biotechnology* 2011, 153, 125-132.

- [53] Focsan, M., Ardelean, II, Craciun, C., Astilean, S., Interplay between gold nanoparticle biosynthesis and metabolic activity of cyanobacterium *Synechocystis* sp. PCC 6803. *Nanotechnology* 2011, 22, 485101.
- [54] Srivastava, S. K., Constanti, M., Room temperature biogenic synthesis of multiple nanoparticles (Ag, Pd, Fe, Rh, Ni, Ru, Pt, Co, and Li) by *Pseudomonas aeruginosa* SM1. *Journal of Nanoparticle Research* 2012, 14, 1-10.
- [55] Kolinko, I., Lohsze, A., Borg, S., Raschdorf, O., *et al.*, Biosynthesis of magnetic nanostructures in a foreign organism by transfer of bacterial magnetosome gene clusters. *Nat Nano* 2014, 9, 193-197.
- [56] Park, T. J., Lee, S. Y., Heo, N. S., Seo, T. S., In vivo synthesis of diverse metal nanoparticles by recombinant *Escherichia coli*. *Angewandte Chemie* 2010, 49, 7019-7024.
- [57] Plaza, D. O., Gallardo, C., Straub, Y. D., Bravo, D., Pérez-Donoso, J. M., Biological synthesis of fluorescent nanoparticles by cadmium and tellurite resistant Antarctic bacteria: exploring novel natural nanofactories. *Microbial Cell Factories* 2016, 15, 1-11.
- [58] Bai, H.-J., Yang, B.-S., Chai, C.-J., Yang, G.-E., *et al.*, Green synthesis of silver nanoparticles using *Rhodobacter Sphaeroides*. *World Journal of Microbiology and Biotechnology* 2011, 27, 2723-2728.
- [59] Lok, C. N., Ho, C. M., Chen, R., Tam, P. K., *et al.*, Proteomic identification of the Cus system as a major determinant of constitutive *Escherichia coli* silver resistance of chromosomal origin. *J Proteome Res* 2008, 7, 2351-2356.
- [60] Liu, J., Zang, L., Wang, Y., Liu, G., Preparation of acridine orange-doped silica nanoparticles for pH measurement. *Journal of Luminescence* 2014, 147, 155-158.
- [61] Shi, L., Squier, T. C., Zachara, J. M., Fredrickson, J. K., Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multiheme c-type cytochromes. *Mol Microbiol* 2007, 65, 12-20.
- [62] Suresh, A. K., Pelletier, D. A., Wang, W., Moon, J. W., *et al.*, Silver nanocrystallites: biofabrication using *Shewanella oneidensis*, and an evaluation of their comparative toxicity on gram-negative and gram-positive bacteria. *Environ Sci Technol* 2010, 44, 5210-5215.
- [63] Ramanathan, R., Field, M. R., O'Mullane, A. P., Smooker, P. M., *et al.*, Aqueous phase synthesis of copper nanoparticles: a link between heavy metal resistance and nanoparticle synthesis ability in bacterial systems. *Nanoscale* 2013, 5, 2300-2306.
- [64] Lowenstam, H., Minerals formed by organisms. *Science* 1981, 211, 1126-1131.
- [65] Yang, D., Zhou, Y., Rui, X., Zhu, J., *et al.*, Fe₃O₄ nanoparticle chains with N-doped carbon coating: magnetotactic bacteria assisted synthesis and high-rate lithium storage. *RSC Advances* 2013, 3, 14960.
- [66] Hershey, D. M., Ren, X., Melnyk, R. A., Browne, P. J., *et al.*, MamO Is a Repurposed Serine Protease that Promotes Magnetite Biomineralization through Direct Transition Metal Binding in Magnetotactic Bacteria. *PLoS Biol* 2016, 14, e1002402.
- [67] Zhang, X. Y., Robledo, B. N., Harris, S. S., Hu, X. P., A bacterial gene, *mms6*, as a new reporter gene for magnetic resonance imaging of mammalian cells. *Mol Imaging* 2014, 13.

- [68] Tandeau de Marsac, N., Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. *FEMS Microbiology Letters* 1993, *104*, 119-189.
- [69] Patel, V., Berthold, D., Puranik, P., Gantar, M., Screening of cyanobacteria and microalgae for their ability to synthesize silver nanoparticles with antibacterial activity. *Biotechnology Reports* 2015, *5*, 112-119.
- [70] Birla, S. S., Gaikwad, S. C., Gade, A. K., Rai, M. K., Rapid synthesis of silver nanoparticles from *Fusarium oxysporum* by optimizing physiocultural conditions. *ScientificWorldJournal* 2013, *2013*, 796018.
- [71] Xie, J., Lee, J. Y., Wang, D. I., Ting, Y. P., Identification of active biomolecules in the high-yield synthesis of single-crystalline gold nanoplates in algal solutions. *Small* 2007, *3*, 672-682.
- [72] Delalat, B., Sheppard, V. C., Rasi Ghaemi, S., Rao, S., *et al.*, Targeted drug delivery using genetically engineered diatom biosilica. *Nature communications* 2015, *6*, 8791.
- [73] Sathish Kumar, K., Amutha, R., Arumugam, P., Berchmans, S., Synthesis of gold nanoparticles: an ecofriendly approach using *Hansenula anomala*. *ACS applied materials & interfaces* 2011, *3*, 1418-1425.
- [74] Patel, N., Kaler, A., Jain, S., Banerjee, U. C., Biosynthesis of Selenium Nanoparticle by Whole Cells of *Saccharomyces boulardii* and its Evaluation as Anticancer Agent. *Current Nanoscience* 2013, *9*, 463-468.
- [75] Salvadori, M. R., Ando, R. A., Oller do Nascimento, C. A., Correa, B., Intracellular biosynthesis and removal of copper nanoparticles by dead biomass of yeast isolated from the wastewater of a mine in the Brazilian Amazonia. *PloS one* 2014, *9*, e87968.
- [76] Jeong, C. K., Kim, I., Park, K.-I., Oh, M. H., *et al.*, Virus-Directed Design of a Flexible BaTiO₃ Nanogenerator. *ACS Nano* 2013, *7*, 11016–11025.
- [77] Ruffing, A. M., Jensen, T. J., Strickland, L. M., Genetic tools for advancement of *Synechococcus* sp. PCC 7002 as a cyanobacterial chassis. *Microb Cell Fact* 2016, *15*, 190.
- [78] Murray, J. M., Watson, A. T., Carr, A. M., Molecular Genetic Tools and Techniques in Fission Yeast. *Cold Spring Harb Protoc* 2016, *2016*, pdb top087601.
- [79] Kellner, N., Schwarz, J., Sturm, M., Fernandez-Martinez, J., *et al.*, Developing genetic tools to exploit *Chaetomium thermophilum* for biochemical analyses of eukaryotic macromolecular assemblies. *Sci Rep* 2016, *6*, 20937.
- [80] Hulkoti, N. I., Taranath, T. C., Biosynthesis of nanoparticles using microbes- a review. *Colloids Surf B Biointerfaces* 2014, *121*, 474-483.
- [81] Lee, S. Y., Kim, H. U., Systems strategies for developing industrial microbial strains. *Nat Biotechnol* 2015, *33*, 1061-1072.
- [82] Mazzola, L., Commercializing Nanotechnology. *Nature Biotechnology* 2003, *21*, 1137-1143.
- [83] Mensah, T. O., Wang, B., Bothun, G., Winter, J., Davis, V., Nanotechnology Commercialization: Manufacturing Processes and Products. Wiley 2017.
- [84] Moon, J. W., Roh, Y., Lauf, R. J., Vali, H., *et al.*, Microbial preparation of metal-substituted magnetite nanoparticles. *Journal of microbiological methods* 2007, *70*, 150-158.

- [85] Lloyd, J. R., Byrne, J. M., Coker, V. S., Biotechnological synthesis of functional nanomaterials. *Current opinion in biotechnology* 2011, 22, 509-515.
- [86] Ferrari, M., Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer* 2005, 5, 161-171.
- [87] Peer, D., Karp, J. M., Hong, S., Farokhzad, O. C., *et al.*, Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007, 2, 751-760.
- [88] Shi, J., Votruba, A. R., Farokhzad, O. C., Langer, R., Nanotechnology in drug delivery and tissue engineering: from discovery to applications. *Nano Lett* 2010, 10, 3223-3230.
- [89] Kearney, C. J., Mooney, D. J., Macroscale delivery systems for molecular and cellular payloads. *Nat Mater* 2013, 12, 1004-1017.
- [90] Stathopoulos, G. P., Antoniou, D., Dimitroulis, J., Michalopoulou, P., *et al.*, Liposomal cisplatin combined with paclitaxel versus cisplatin and paclitaxel in non-small-cell lung cancer: a randomized phase III multicenter trial. *Ann Oncol* 2010, 21, 2227-2232.
- [91] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01644890?term> (2016).
- [92] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT00377936?term> (2008).
- [93] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT00542048?term> (2010).
- [94] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01537536?term>.
- [95] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT00448305?term>.
- [96] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02494570?term>.
- [97] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02646319?term>.
- [98] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01380769?term>.
- [99] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02187302?term>.
- [100] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01652079?term>.
- [101] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02213744?term>.
- [102] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01792479?term>.
- [103] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02283320?term>.
- [104] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01812746?term>.
- [105] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT00964080?term>.

- [106] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01702129?term>.
- [107] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT02112656?term>.
- [108] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT01696084?term>.
- [109] US National Library of Medicine. ClinicalTrials.gov (2018, May 27) retrieved from <https://clinicaltrials.gov/ct2/show/NCT00361842?term>.
- [110] Matsumura, Y., Maeda, H., A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer research* 1986, *46*, 6387-6392.
- [111] Gerlowski, L. E., Jain, R. K., Microvascular permeability of normal and neoplastic tissues. *Microvascular Research* 1986, *31*, 288-305.
- [112] Bertrand, N., Wu, J., Xu, X., Kamaly, N., Farokhzad, O. C., Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. *Adv Drug Deliv Rev* 2014, *66*, 2-25.
- [113] Maeda, H., Toward a full understanding of the EPR effect in primary and metastatic tumors as well as issues related to its heterogeneity. *Adv Drug Deliv Rev* 2015, *91*, 3-6.
- [114] Stephen, Z. R., Kievit, F. M., Zhang, M., Magnetite nanoparticles for medical MR imaging. *Materials Today* 2011, *14*, 330-338.
- [115] Shin, T. H., Choi, Y., Kim, S., Cheon, J., Recent advances in magnetic nanoparticle-based multi-modal imaging. *Chem Soc Rev* 2015, *44*, 4501-4516.
- [116] Estelrich, J., Sanchez-Martin, M. J., Busquets, M. A., Nanoparticles in magnetic resonance imaging: from simple to dual contrast agents. *Int J Nanomedicine* 2015, *10*, 1727-1741.
- [117] Wieggers, C. B., Welch, M. J., Sharp, T. L., Brown, J. J., *et al.*, Evaluation of two new gadolinium chelates as contrast agents for MRI. *Magn Reson Imaging* 1992, *10*, 903-911.
- [118] Lee, G. H., Chang, Y., Kim, T.-J., Blood-Pool and Targeting MRI Contrast Agents: From Gd-Chelates to Gd-Nanoparticles. *European Journal of Inorganic Chemistry* 2012, *2012*, 1924-1933.
- [119] Hashim, Z., Green, M., Chung, P. H., Suhling, K., *et al.*, Gd-containing conjugated polymer nanoparticles: bimodal nanoparticles for fluorescence and MRI imaging. *Nanoscale* 2014, *6*, 8376-8386.
- [120] Jennings, L. E., Long, N. J., 'Two is better than one'--probes for dual-modality molecular imaging. *Chem Commun (Camb)* 2009, 3511-3524.
- [121] Rumenapp, C., Gleich, B., Haase, A., Magnetic nanoparticles in magnetic resonance imaging and diagnostics. *Pharm Res* 2012, *29*, 1165-1179.
- [122] Winter, P., Athey, P., Kiefer, G., Gulyas, G., *et al.*, Improved paramagnetic chelate for molecular imaging with MRI. *Journal of Magnetism and Magnetic Materials* 2005, *293*, 540-545.
- [123] Frias, J. C., Williams, K. J., Fisher, E. A., Fayad, Z. A., Recombinant HDL-like nanoparticles: a specific contrast agent for MRI of atherosclerotic plaques. *Journal of the American Chemical Society* 2004, *126*, 16316-16317.
- [124] European Medicines Agency. EMA/486286/2012. London, UK: European

Medicines Agency; 2012.

[125] Pan, D., Caruthers, S. D., Senpan, A., Schmieder, A. H., *et al.*, Revisiting an old friend: manganese-based MRI contrast agents. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2011, 3, 162-173.

[126] Filippousi, M., Angelakeris, M., Katsikini, M., Paloura, E., *et al.*, Surfactant Effects on the Structural and Magnetic Properties of Iron Oxide Nanoparticles. *The Journal of Physical Chemistry C* 2014, 118, 16209-16217.

[127] Shokrollahi, H., Structure, synthetic methods, magnetic properties and biomedical applications of ferrofluids. *Mater Sci Eng C Mater Biol Appl* 2013, 33, 2476-2487.

[128] Pflipsen, C., Forge, D., Benali, S., Gossuin, Y., Improved Stability and Relaxivity of a Commercial Magnetic Ferrofluid. *The Journal of Physical Chemistry C* 2013, 117, 20919-20926.

[129] Bulte, J. W., Kraitchman, D. L., Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed* 2004, 17, 484-499.

[130] Sana, B., Johnson, E., Sheah, K., Poh, C. L., Lim, S., Iron-based ferritin nanocore as a contrast agent. *Biointerphases* 2010, 5, FA48-52.

[131] Aime, S., Frullano, L., Geninatti Crich, S., Compartmentalization of a Gadolinium Complex in the Apoferritin Cavity: A Route To Obtain High Relaxivity Contrast Agents for Magnetic Resonance Imaging. *Angewandte Chemie International Edition* 2002, 41, 1017-1019.

[132] Torchilin, V. P., Liposomes as delivery agents for medical imaging. *Molecular Medicine Today* 1996, 2, 242-249.

[133] Aime, S., Delli Castelli, D., Terreno, E., Highly sensitive MRI chemical exchange saturation transfer agents using liposomes. *Angewandte Chemie* 2005, 44, 5513-5515.

[134] Kamaly, N., Miller, A. D., Paramagnetic liposome nanoparticles for cellular and tumour imaging. *Int J Mol Sci* 2010, 11, 1759-1776.

[135] Ruder, W. C., Lu, T., Collins, J. J., Synthetic biology moving into the clinic. *Science* 2011, 333, 1248-1252.

[136] Elowitz, M. B., Leibler, S., A synthetic oscillatory network of transcriptional regulators. *Nature* 2000, 403, 335-338.

[137] Gardner, T. S., Cantor, C. R., Collins, J. J., Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 2000, 403, 339-342.

[138] Friedland, A. E., Lu, T. K., Wang, X., Shi, D., *et al.*, Synthetic gene networks that count. *Science* 2009, 324, 1199-1202.

[139] Kapitein, L. C., Peterman, E. J., Kwok, B. H., Kim, J. H., *et al.*, The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* 2005, 435, 114-118.

[140] Rinaudo, K., Bleris, L., Maddamsetti, R., Subramanian, S., *et al.*, A universal RNAi-based logic evaluator that operates in mammalian cells. *Nat Biotechnol* 2007, 25, 795-801.

[141] Danino, T., Mondragon-Palomino, O., Tsimring, L., Hasty, J., A synchronized quorum of genetic clocks. *Nature* 2010, 463, 326-330.

- [142] Ellis, T., Wang, X., Collins, J. J., Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nat Biotechnol* 2009, 27, 465-471.
- [143] You, L., Cox, R. S., 3rd, Weiss, R., Arnold, F. H., Programmed population control by cell-cell communication and regulated killing. *Nature* 2004, 428, 868-871.
- [144] Folcher, M., Oesterle, S., Zwicky, K., Thekkotttil, T., *et al.*, Mind-controlled transgene expression by a wireless-powered optogenetic designer cell implant. *Nat Commun* 2014, 5, 5392.
- [145] Anderson, J. C., Clarke, E. J., Arkin, A. P., Voigt, C. A., Environmentally controlled invasion of cancer cells by engineered bacteria. *Journal of molecular biology* 2006, 355, 619-627.
- [146] Xiang, S., Fruehauf, J., Li, C. J., Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nat Biotechnol* 2006, 24, 697-702.
- [147] Critchley, R. J., Jezard, S., Vassaux, G., Genetically Engineered E. coli as a Protein Delivery Vehicle for Killing Cancer Cells. *Discovery Medicine* 2004, 4, 194-197.
- [148] Johnson, L., Shen, A., Boyle, L., Kunich, J., *et al.*, Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell* 2002, 1, 325-337.
- [149] Kirn, D. H., Thorne, S. H., Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer* 2009, 9, 64-71.
- [150] Breitbach, C. J., Burke, J., Jonker, D., Stephenson, J., *et al.*, Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 2011, 477, 99-102.
- [151] Fernandez-Garcia, M., Martinez-Arias, A., Hanson, J. C., Rodriguez, J. A., Nanostructured oxides in chemistry: characterization and properties. *Chemical reviews* 2004, 104, 4063-4104.
- [152] Hyeon, T., Lee, S. S., Park, J., Chung, Y., Na, H. B., Synthesis of highly crystalline and monodisperse maghemite nanocrystallites without a size-selection process. *Journal of the American Chemical Society* 2001, 123, 12798-12801.
- [153] Abhilash, Revati, K., Pandey, B. D., Microbial synthesis of iron-based nanomaterials—A review. *Bull Mater Sci* 2011, 34, 191-198.
- [154] Meldrum, F. C., Wade, V. J., Nimmo, D. L., Heywood, B. R., Mann, S., Synthesis of inorganic nanophase materials in supramolecular protein cages. *Nature* 1991, 349, 684-687.
- [155] Blum, A. S., Soto, C. M., Wilson, C. D., Cole, J. D., *et al.*, Cowpea Mosaic Virus as a Scaffold for 3-D Patterning of Gold Nanoparticles. *Nano Letters* 2004, 4, 413-417.
- [156] Andrews, S. C., Robinson, A. K., Rodriguez-Quinones, F., Bacterial iron homeostasis. *FEMS Microbiology Reviews* 2003, 27, 215-237.
- [157] Köster, W., ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Research in Microbiology* 2001, 152, 291-301.
- [158] Meike Kammler, Claudia Schon, Hantke, K., Characterization of the ferrous iron uptake system of Escherichia coli. *Journal of Bacteriology* 1993, 175, 6212-6219.

- [159] Stojiljkovic, I., Cobeljic, M., Hantke, K., Escherichia coli K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. *FEMS Microbiology Letters* 1993, *108*, 111-115.
- [160] Cowart, R. E., Reduction of iron by extracellular iron reductases- implications for microbial iron acquisition. *Archives of Biochemistry and Biophysics* 2002, *400*, 273-281.
- [161] Andrews, S. C., Iron storage in bacteria. *Advances in microbial physiology* 1998, *40*, 281-351.
- [162] Dautant, A., Meyer, J. B., Yariv, J., Precigoux, G., *et al.*, Structure of a monoclinic crystal from of cytochrome b1 (Bacterioferritin) from E. coli. *Acta crystallographica. Section D, Biological crystallography* 1998, *54*, 16-24.
- [163] R.A. Grant, Filman, D. J., Finkel, S. E., Kolter, R., Hogle, J. M., The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nature Structural Biology* 1998, *5*, 294-393.
- [164] Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., *et al.*, Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of Escherichia coli. *The Journal of biological chemistry* 2002, *277*, 27689-27696.
- [165] Johnson, E., Cascio, D., Sawaya, M. R., Gingery, M., Schroder, I., Crystal structures of a tetrahedral open pore ferritin from the hyperthermophilic archaeon Archaeoglobus fulgidus. *Structure* 2005, *13*, 637-648.
- [166] Matsunaga, T., Suzuki, T., Tanaka, M., Arakaki, A., Molecular analysis of magnetotactic bacteria and development of functional bacterial magnetic particles for nano-biotechnology. *Trends Biotechnol* 2007, *25*, 182-188.
- [167] Arakaki, A., Webb, J., Matsunaga, T., A novel protein tightly bound to bacterial magnetic particles in Magnetospirillum magneticum strain AMB-1. *The Journal of biological chemistry* 2003, *278*, 8745-8750.
- [168] Prozorov, T., Mallapragada, S. K., Narasimhan, B., Wang, L., *et al.*, Protein-Mediated Synthesis of Uniform Superparamagnetic Magnetite Nanocrystals. *Advanced Functional Materials* 2007, *17*, 951-957.
- [169] Arakaki, A., Masuda, F., Amemiya, Y., Tanaka, T., Matsunaga, T., Control of the morphology and size of magnetite particles with peptides mimicking the Mms6 protein from magnetotactic bacteria. *J Colloid Interface Sci* 2010, *343*, 65-70.
- [170] Hantke, K., Ferrous iron transport mutants in Escherichia coli K12. *FEMS Microbiology Letters* 1987, *44*, 53-57.
- [171] Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J., Andrews, S. C., Feo--transport of ferrous iron into bacteria. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2006, *19*, 143-157.
- [172] Marlovits, T. C., Haase, W., Herrmann, C., Aller, S. G., Unger, V. M., The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 2002, *99*, 16243-16248.
- [173] Nies, D. H., Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews* 2003, *27*, 313-339.

- [174] Grass, G., Otto, M., Fricke, B., Haney, C. J., *et al.*, FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. *Archives of microbiology* 2005, 183, 9-18.
- [175] Datsenko, K. A., Wanner, B. L., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America* 2000, 97, 6640-6645.
- [176] Hantke, K., Iron and metal regulation in bacteria. *Current Opinion in Microbiology* 2001, 4, 172-177.
- [177] Escolar, L., Pérez-Martín, J., de Lorenzo, V., Opening the Iron Box: Transcriptional Metalloregulation by the Fur Protein. *Journal of Bacteriology* 1999, 181, 6223-6229.
- [178] Woodmansee, A. N., Imlay, J. A., Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. *The Journal of biological chemistry* 2002, 277, 34055-34066.
- [179] Kumar, K. S., Pasula, R. R., Lim, S., Nijhuis, C. A., Long-Range Tunneling Processes across Ferritin-Based Junctions. *Adv Mater* 2016, 28, 1824-1830.
- [180] Caforio, A., Siliakus, M. F., Exterkate, M., Jain, S., *et al.*, Converting *Escherichia coli* into an archaeobacterium with a hybrid heterochiral membrane. *Proceedings of the National Academy of Sciences of the United States of America* 2018, 115, 3704-3709.
- [181] Gubellini, F., Verdon, G., Karpowich, N. K., Luff, J. D., *et al.*, Physiological response to membrane protein overexpression in *E. coli*. *Mol Cell Proteomics* 2011, 10, M111 007930.
- [182] Chasteen, N. D., Harrison, P. M., Mineralization in ferritin : an efficient means of iron storage. *Journal of Structural Biology* 1999, 126, 182-194.
- [183] Babay, S., Mhiri, T., Toumi, M., Synthesis, structural and spectroscopic characterizations of maghemite γ -Fe₂O₃ prepared by one-step coprecipitation route. *Journal of Molecular Structure* 2015, 1085, 286-293.
- [184] Teja, A. S., Koh, P.-Y., Synthesis, properties, and applications of magnetic iron oxide nanoparticles. *Progress in Crystal Growth and Characterization of Materials* 2009, 55, 22-45.
- [185] Machala, L., Tuček, J. i., Zbořil, R., Polymorphous Transformations of Nanometric Iron(III) Oxide: A Review. *Chemistry of Materials* 2011, 23, 3255-3272.
- [186] Zboril, R., Mashlan, M., Petridis, D., Iron(III) Oxides from Thermal Processes Synthesis, Structural and Magnetic Properties, Mössbauer Spectroscopy Characterization, and Applications†. *Chemistry of Materials* 2002, 14, 969-982.
- [187] Xu, H., Wang, X., Zhang, L., Selective preparation of nanorods and micro-octahedrons of Fe₂O₃ and their catalytic performances for thermal decomposition of ammonium perchlorate. *Powder Technology* 2008, 185, 176-180.
- [188] Cornell, R. M., Schwertmann, U., The iron oxides: structure, properties, reactions, occurrences and uses. *Weinheim: Wiley-VCH* 2003.
- [189] Yan, L., Zhang, S., Chen, P., Liu, H., *et al.*, Magnetotactic bacteria, magnetosomes and their application. *Microbiol Res* 2012, 167, 507-519.
- [190] Bhowmik, R. N., Satya, A. T., Bharathi, A., Synthesis of Co_{1.5}Fe_{1.5}O₄ spinel ferrite with high magnetic squareness and study its magnetic property by annealing

the chemical routed sample at different temperatures. *Journal of Alloys and Compounds* 2013, 559, 134-141.

[191] Ohkoshi, S., Namai, A., Imoto, K., Yoshikiyo, M., *et al.*, Nanometer-size hard magnetic ferrite exhibiting high optical-transparency and nonlinear optical-magnetoelectric effect. *Sci Rep* 2015, 5, 14414.

[192] Halliday, J. W., Powell, L. W., Ferritin metabolism and the liver. *Semin Liver Dis* 1984, 4, 207-216.

[193] Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., *et al.*, Ferritin: Design and Formation of an Iron-Storage Molecule. *Philosophical Transactions of the Royal Society B: Biological Sciences* 1984, 304, 551-565.

[194] Theil, E. C., Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem* 1987, 56, 289-315.

[195] Holden, J., Adams, M. W. W., Microbe-metal interactions in marine hydrothermal environments. *Current Opinion in Chemical Biology* 2003, 7, 160-165.

[196] Le Brun, N. E., Crow, A., Murphy, M. E., Mauk, A. G., Moore, G. R., Iron core mineralisation in prokaryotic ferritins. *Biochim Biophys Acta* 2010, 1800, 732-744.

[197] Sana, B., Johnson, E., Le Magueres, P., Criswell, A., *et al.*, The role of nonconserved residues of *Archaeoglobus fulgidus* ferritin on its unique structure and biophysical properties. *The Journal of biological chemistry* 2013, 288, 32663-32672.

[198] Jutz, G., van Rijn, P., Santos Miranda, B., Boker, A., Ferritin: a versatile building block for bionanotechnology. *Chemical reviews* 2015, 115, 1653-1701.

[199] Jaaskelainen, A., Harinen, R. R., Lamminmaki, U., Korpimaki, T., *et al.*, Production of apoferritin-based bioinorganic hybrid nanoparticles by bacterial fermentation followed by self-assembly. *Small* 2007, 3, 1362-1367.

[200] Pead, S., Durrant, E., Webb, B., Larsen, C., *et al.*, Metal ion binding to apo, holo, and reconstituted horse spleen ferritin. *Journal of Inorganic Biochemistry* 1995, 59, 15-27.

[201] Tosha, T., Ng, H. L., Bhattasali, O., Alber, T., Theil, E. C., Moving metal ions through ferritin-protein nanocages from three-fold pores to catalytic sites. *Journal of the American Chemical Society* 2010, 132, 14562-14569.

[202] Polanams, J., Ray, A. D., Watt, R. K., Nanophase iron phosphate, iron arsenate, iron vanadate, and iron molybdate minerals synthesized within the protein cage of ferritin. *Inorg Chem* 2005, 44, 3203-3209.

[203] Meldrum, F. C., Douglas, T., Levi, S., Arosio, P., Mann, S., Reconstitution of manganese oxide cores in horse spleen and recombinant ferritins. *J Inorg Biochem* 1995, 58, 59-68.

[204] Pulsipher, K. W., Dmochowski, I. J., Ferritin Encapsulation and Templated Synthesis of Inorganic Nanoparticles, in: Orner, B. P. (Ed.), *Protein Cages: Methods and Protocols*, Springer New York, New York, NY 2015, pp. 27-37.

[205] Sana, B., Poh, C. L., Lim, S., A manganese-ferritin nanocomposite as an ultrasensitive T2 contrast agent. *Chem Commun (Camb)* 2012, 48, 862-864.

[206] Sana, B., Calista, M., Lim, S., Protein cage assisted metal-protein nanocomposite synthesis: Optimization of loading conditions. 2012, 82-96.

[207] Savitzky, A., Golay, M. J. E., Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Analytical Chemistry* 1964, 36, 1627-1639.

- [208] Perez-Iratxeta, C., Andrade-Navarro, M. A., K2D2: estimation of protein secondary structure from circular dichroism spectra. *BMC Struct Biol* 2008, 8, 25.
- [209] Greenfield, N. J., Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 2006, 1, 2876-2890.
- [210] Terry L Riss, Moravec, R. A., Andrew L Niles, Sarah Duellman, *et al.*, Eli Lilly & Company and the National Center for Advancing Translational Sciences 2004-.
- [211] Coats, A. W., Redfern, J. P., Thermogravimetric analysis. A review. *The Analyst* 1963, 88, 906.
- [212] Fernandes, L. L., Resende, C. X., Tavares, D. S., Soares, G. A., *et al.*, Cytocompatibility of chitosan and collagen-chitosan scaffolds for tissue engineering. *Polímeros* 2011, 21, 1-6.
- [213] Samouillan, V., Dandurand-Lods, J., Lamure, A., Maurel, E., *et al.*, Thermal analysis characterization of aortic tissues for cardiac valve bioprotheses. *Journal of Biomedical Materials Research* 1999, 46, 531-538.
- [214] Sionkowska, A., Skopinska-Wisniewska, J., Gawron, M., Kozłowska, J., Planecka, A., Chemical and thermal cross-linking of collagen and elastin hydrolysates. *Int J Biol Macromol* 2010, 47, 570-577.
- [215] Dandurand, J., Samouillan, V., Lacoste-Ferre, M. H., Lacabanne, C., *et al.*, Conformational and thermal characterization of a synthetic peptidic fragment inspired from human tropoelastin: Signature of the amyloid fibers. *Pathol Biol (Paris)* 2014, 62, 100-107.
- [216] Kelly, S. M., Jess, T. J., Price, N. C., How to study proteins by circular dichroism. *Biochim Biophys Acta* 2005, 1751, 119-139.
- [217] Berova, N., Nakanishi, K., Woody, R. W., Circular Dichroism. Principles and Applications *VCH Publishers, New York* 2000, 44, 877.
- [218] Barrow, C. J., Yasuda, A., Kenny, P. T., Zagorski, M. G., Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *Journal of molecular biology* 1992, 225, 1075-1093.
- [219] Cheung-Lau, J. C., Liu, D., Pulsipher, K. W., Liu, W., Dmochowski, I. J., Engineering a well-ordered, functional protein-gold nanoparticle assembly. *J Inorg Biochem* 2014, 130, 59-68.
- [220] Haris, P. I., Characterization of Protein Structure and Stability Using Fourier Transform Infrared Spectroscopy. *Pharmacy and Pharmacology Communications* 1999, 5, 15-25.
- [221] Byler, D. M., Susi, H., Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* 1986, 25, 469-487.
- [222] Haris, P. I., Chapman, D., Does Fourier-Transform infrared spectroscopy provide useful information on protein structures? *Trends in Biochemical Sciences* 1992, 17, 328-333.
- [223] Surewicz, W. K., Mantsch, H. H., New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1988, 952, 115-130.

- [224] Morawski, A. M., Winter, P. M., Crowder, K. C., Caruthers, S. D., *et al.*, Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI. *Magn Reson Med* 2004, *51*, 480-486.
- [225] Raji, M. A., Amara, M., Amoabediny, G., Tajik, P., *et al.*, Cytotoxicity of synthesized Iron Oxide nanoparticles: toward novel biomarkers of colon cancer. *Conf Proc IEEE Eng Med Biol Soc* 2014, *2014*, 6179-6182.
- [226] Liu, X., Wei, W., Yuan, Q., Zhang, X., *et al.*, Apoferritin-CeO₂ nano-truffle that has excellent artificial redox enzyme activity. *Chem Commun (Camb)* 2012, *48*, 3155-3157.
- [227] Liu, X., Wei, W., Wang, C., Yue, H., *et al.*, Apoferritin-camouflaged Pt nanoparticles: surface effects on cellular uptake and cytotoxicity. *Journal of Materials Chemistry* 2011, *21*, 7105.
- [228] Nandwana, V., Ryoo, S. R., Kanthala, S., Kumar, A., *et al.*, Engineered ferritin nanocages as natural contrast agents in magnetic resonance imaging. *RSC Advances* 2017, *7*, 34892-34900.
- [229] Dougherty, T. J., Photochemistry in the Treatment of Cancer. 1992, 275-311.
- [230] Kalka, K., Merk, H., Mukhtar, H., Photodynamic therapy in dermatology. *Journal of the American Academy of Dermatology* 2000, *42*, 389-413.
- [231] Ochsner, M., Photophysical and photobiological processes in the photodynamic therapy of tumours. *Journal of Photochemistry and Photobiology B: Biology* 1997, *39*, 1-18.
- [232] Van Hillegersberg, R., Hekking-Weijma, J. M., Wilson, J. H. P., Edixhoven-Bosdijk, A., Kort, W. J., Adjuvant intraoperative photodynamic therapy diminishes the rate of local recurrence in a rat mammary tumour model. *British Journal of Cancer* 1995, *71*, 733-737.
- [233] Haddad, R., Effect of photodynamic therapy on normal fibroblasts and colon anastomotic healing in mice. *Journal of Gastrointestinal Surgery* 1999, *3*, 602-606.
- [234] Tardivo, J. P., Del Giglio, A., Paschoal, L. H. C., Ito, A. S., Baptista, M. S., Treatment of melanoma lesions using methylene blue and RL50 light source. *Photodiagnosis and Photodynamic Therapy* 2004, *1*, 345-346.
- [235] Huang, Z., Hsu, Y.-C., Li, L.-B., Wang, L.-W., *et al.*, Photodynamic therapy of cancer — Challenges of multidrug resistance. *Journal of Innovative Optical Health Sciences* 2015, *08*, 1530002.
- [236] Yoon, I., Li, J. Z., Shim, Y. K., Advance in Photosensitizers and Light Delivery for Photodynamic Therapy. *Clinical Endoscopy* 2013, *46*, 7.
- [237] Plaetzer, K., Kiesslich, T., Oberdanner, C., Krammer, B., Apoptosis Following Photodynamic Tumor Therapy: Induction, Mechanisms and Detection. *Current Pharmaceutical Design* 2005, *11*, 1151-1165.
- [238] Lemasters, J. J., Dying a Thousand Deaths: Redundant Pathways From Different Organelles to Apoptosis and Necrosis. *Gastroenterology* 2005, *129*, 351-360.
- [239] Kochevar, I. E., Lynch, M. C., Zhuang, S., Lambert, C. R., Singlet Oxygen, but not Oxidizing Radicals, Induces Apoptosis in HL-60 Cells. *Photochemistry and Photobiology* 2007, *72*, 548-553.

- [240] Almeida, R. D., Manadas, B. J., Carvalho, A. P., Duarte, C. B., Intracellular signaling mechanisms in photodynamic therapy. *Biochim Biophys Acta* 2004, 1704, 59-86.
- [241] Mellish, K. J., Cox, R. D., Vernon, D. I., Griffiths, J., Brown, S. B., In Vitro Photodynamic Activity of a Series of Methylene Blue Analogues¶. *Photochemistry and Photobiology* 2007, 75, 392-397.
- [242] Kristjan, P., Tobias, K., Christian Benno, O., Barbara, K., Apoptosis Following Photodynamic Tumor Therapy: Induction, Mechanisms and Detection. *Current Pharmaceutical Design* 2005, 11, 1151-1165.
- [243] Kessel, D., Luo, Y., Deng, Y., Chang, C. K., The Role of Subcellular Localization in Initiation of Apoptosis by Photodynamic Therapy. *Photochemistry and Photobiology* 1997, 65, 422-426.
- [244] Trindade, G. S., Farias, S. L. A., Rumjanek, V. M., Capella, M. A. M., Methylene blue reverts multidrug resistance: sensitivity of multidrug resistant cells to this dye and its photodynamic action. *Cancer Letters* 2000, 151, 161-167.
- [245] Kessel, D., Luo, Y., Mitochondrial photodamage and PDT-induced apoptosis. *Journal of Photochemistry and Photobiology B: Biology* 1998, 42, 89-95.
- [246] Kandela, I. K., Bartlett, J. A., Indig, G. L., Effect of molecular structure on the selective phototoxicity of triarylmethane dyes towards tumor cells. *Photochemical & Photobiological Sciences* 2002, 1, 309-314.
- [247] Kowaltowski, A. J., Turin, J., Indig, G. L., Vercesi, A. E., Mitochondrial effects of triarylmethane dyes. *Journal of bioenergetics and biomembranes* 1999, 31, 581-590.
- [248] Foote, C. S., Mechanisms of Photosensitized Oxidation. *Science* 1968, 162, 963-970.
- [249] Juarranz, Á., Jaén, P., Sanz-Rodríguez, F., Cuevas, J., González, S., Photodynamic therapy of cancer. Basic principles and applications. *Clinical and Translational Oncology* 2008, 10, 148-154.
- [250] Moore, J. V., Photodynamic Therapy: Basic Principles and Clinical Applications. *British Journal of Cancer* 1993, 68, 1257-1257.
- [251] Dougherty, T. J., Grindey, G. B., Fiel, R., Weishaupt, K. R., Boyle, D. G., Photoradiation Therapy. II. Cure of Animal Tumors With Hematoporphyrin and Light. *Journal of the National Cancer Institute* 1975, 55, 115-121.
- [252] Brown, S. B., Brown, E. A., Walker, I., The present and future role of photodynamic therapy in cancer treatment. *The Lancet Oncology* 2004, 5, 497-508.
- [253] Via, L., Magno, S., Photochemotherapy in the Treatment of Cancer. *Current Medicinal Chemistry* 2001, 8, 1405-1418.
- [254] Allison, R. R., Downie, G. H., Cuenca, R., Hu, X.-H., *et al.*, Photosensitizers in clinical PDT. *Photodiagnosis and Photodynamic Therapy* 2004, 1, 27-42.
- [255] O'Connor, A. E., Gallagher, W. M., Byrne, A. T., Porphyrin and nonporphyrin photosensitizers in oncology: preclinical and clinical advances in photodynamic therapy. *Photochem Photobiol* 2009, 85, 1053-1074.
- [256] Allison, R. R., Sibata, C. H., Oncologic photodynamic therapy photosensitizers: a clinical review. *Photodiagnosis Photodyn Ther* 2010, 7, 61-75.

- [257] Xiang, Z., Ravindra, K. P., Porphyrin-Carbohydrate Conjugates: Impact of Carbohydrate Moieties in Photodynamic Therapy (PDT). *Anti-Cancer Agents in Medicinal Chemistry* 2008, 8, 241-268.
- [258] Shadidi, M., Sioud, M., Selective targeting of cancer cells using synthetic peptides. *Drug Resistance Updates*, 6, 363-371.
- [259] Polo, L., Valduga, G., Jori, G., Reddi, E., Low-density lipoprotein receptors in the uptake of tumour photosensitizers by human and rat transformed fibroblasts. *The International Journal of Biochemistry & Cell Biology* 2002, 34, 10-23.
- [260] Vrouenraets, M. B., Visser, G. W. M., Loup, C., Meunier, B., *et al.*, Targeting of a hydrophilic photosensitizer by use of internalizing monoclonal antibodies: A new possibility for use in photodynamic therapy. *International Journal of Cancer* 2000, 88, 108-114.
- [261] Chatterjee, D. K., Fong, L. S., Zhang, Y., Nanoparticles in photodynamic therapy: an emerging paradigm. *Adv Drug Deliv Rev* 2008, 60, 1627-1637.
- [262] Greco, F., Polymer-drug conjugates: current status and future trends. *Frontiers in Bioscience* 2008, 13, 2744.
- [263] Maeda, H., SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. *Adv Drug Deliv Rev* 2001, 46, 169-185.
- [264] Debele, T. A., Peng, S., Tsai, H. C., Drug Carrier for Photodynamic Cancer Therapy. *Int J Mol Sci* 2015, 16, 22094-22136.
- [265] Maeda, H., Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjug Chem* 2010, 21, 797-802.
- [266] Kobayashi, H., Watanabe, R., Choyke, P. L., Improving conventional enhanced permeability and retention (EPR) effects; what is the appropriate target? *Theranostics* 2013, 4, 81-89.
- [267] Felice, B., Prabhakaran, M. P., Rodriguez, A. P., Ramakrishna, S., Drug delivery vehicles on a nano-engineering perspective. *Mater Sci Eng C Mater Biol Appl* 2014, 41, 178-195.
- [268] Lassalle, H. P., Wagner, M., Bezdetnaya, L., Guillemin, F., Schneckenburger, H., Fluorescence imaging of Foscan and Foslip in the plasma membrane and in whole cells. *Journal of photochemistry and photobiology. B, Biology* 2008, 92, 47-53.
- [269] Castano, A. P., Demidova, T. N., Hamblin, M. R., Mechanisms in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization. *Photodiagnosis and Photodynamic Therapy* 2004, 1, 279-293.
- [270] Brandis, A. S., Salomon, Y., Scherz, A., Chlorophyll Sensitizers in Photodynamic Therapy, in: Grimm, B., Porra, R. J., Rüdiger, W., Scheer, H. (Eds.), *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*, Springer Netherlands, Dordrecht 2006, pp. 461-483.
- [271] Kapuscinski, J., Darzynkiewicz, Z., Melamed, M. R., Interactions of acridine orange with nucleic acids. Properties of complexes of acridine orange with single stranded ribonucleic acid. *Biochemical pharmacology* 1983, 32, 3679-3694.
- [272] Korgaonkar, K. S., Sukhatankar, J. V., Anti-Tumour Activity of the Fluorescent Dye, Acridine Orange, on Yoshida Sarcoma (Ascites). *British Journal of Cancer* 1963, 17, 471-473.

- [273] Zdolsek, J. M., Olsson, G. M., Brunk, U. T., Photooxidative damage to lysosomes of cultured macrophages by acridine orange. *Photochem Photobiol* 1990, 51, 67-76.
- [274] Lewis, M. R., Goland, P. P., In vivo staining and retardation of tumors in mice by acridine compounds. *The American journal of the medical sciences* 1948, 215, 282-289.
- [275] Rickman, L. S., Long, G. W., Oberst, R., Cabanban, A., *et al.*, Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. *Lancet (London, England)* 1989, 1, 68-71.
- [276] Zampieri, A., Greenberg, J., Mutagenesis by acridine orange and proflavine in Escherichia coli strain S. *Mutation research* 1965, 2, 552-556.
- [277] McCann, J., Choi, E., Yamasaki, E., Ames, B. N., Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. *Proceedings of the National Academy of Sciences of the United States of America* 1975, 72, 5135-5139.
- [278] Sastry, K. S., Gordon, M. P., The photodynamic inactivation of tobacco mosaic virus and its ribonucleic acid by acridine orange. *Biochim Biophys Acta* 1966, 129, 32-41.
- [279] Amagasa, J., Mechanisms of photodynamic inactivation of acridine orange-sensitized transfer RNA : Participation of singlet oxygen and base damage leading to inactivation. *Journal of Radiation Research* 1986, 27, 339-351.
- [280] Zelenin, A. V., Fluorescence Microscopy of Lysosomes and Related Structures in Living Cells. *Nature* 1966, 212, 425-426.
- [281] Kusuzaki, K., Minami, G., Takeshita, H., Murata, H., *et al.*, Photodynamic inactivation with acridine orange on a multidrug-resistant mouse osteosarcoma cell line. *Japanese journal of cancer research : Gann* 2000, 91, 439-445.
- [282] Kusuzaki, K., Aomori, K., Sugino, T., Minami, G., *et al.*, Total tumor cell elimination with minimum damage to normal tissues in musculoskeletal sarcomas following photodynamic therapy with acridine orange. *Oncology* 2000, 59, 174-180.
- [283] Kusuzaki, K., Matsubara, T., Satonaka, H., Matsumine, A., *et al.*, Intraoperative Photodynamic Surgery (iPDS) with Acridine Orange for Musculoskeletal Sarcomas. *Cureus* 2014.
- [284] MATSUBARA, T., KUSUZAKI, K., MATSUMINE, A., SHINTANI, K., *et al.*, Acridine Orange Used for Photodynamic Therapy Accumulates in Malignant Musculoskeletal Tumors Depending on pH Gradient. *Anticancer research* 2006, 26, 187-193.
- [285] Hashiguchi, S., Kusuzaki, K., Murata, H., Takeshita, H., *et al.*, Acridine orange excited by low-dose radiation has a strong cytotoxic effect on mouse osteosarcoma. *Oncology* 2002, 62, 85-93.
- [286] Pogue, B. W., O'Hara, J. A., Demidenko, E., Wilmot, C. M., *et al.*, Photodynamic therapy with verteporfin in the radiation-induced fibrosarcoma-1 tumor causes enhanced radiation sensitivity. *Cancer research* 2003, 63, 1025-1033.
- [287] Webb, B., Frame, J., Zhao, Z., Lee, M. L., Watt, G. D., Molecular entrapment of small molecules within the interior of horse spleen ferritin. *Arch Biochem Biophys* 1994, 309, 178-183.

- [288] Simsek, E., Akif Kilic, M., Magic ferritin: A novel chemotherapeutic encapsulation bullet. *Journal of Magnetism and Magnetic Materials* 2005, 293, 509-513.
- [289] Aime, S., Frullano, L., Geninatti Crich, S., Compartmentalization of a gadolinium complex in the apoferritin cavity: a route to obtain high relaxivity contrast agents for magnetic resonance imaging. *Angewandte Chemie* 2002, 41, 1017-1019.
- [290] Hennequin, B., Turyanska, L., Ben, T., Beltrán, A. M., *et al.*, Aqueous Near-Infrared Fluorescent Composites Based on Apoferritin-Encapsulated PbS Quantum Dots. *Advanced Materials* 2008, 20, 3592-3596.
- [291] Yan, F., Zhang, Y., Kim, K. S., Yuan, H. K., Vo-Dinh, T., Cellular uptake and photodynamic activity of protein nanocages containing methylene blue photosensitizing drug. *Photochem Photobiol* 2010, 86, 662-666.
- [292] Zhen, Z., Tang, W., Guo, C., Chen, H., *et al.*, Ferritin nanocages to encapsulate and deliver photosensitizers for efficient photodynamic therapy against cancer. *ACS Nano* 2013, 7, 6988-6996.
- [293] Yan, F., Zhang, Y., Yuan, H. K., Gregas, M. K., Vo-Dinh, T., Apoferritin protein cages: a novel drug nanocarrier for photodynamic therapy. *Chem Commun (Camb)* 2008, 4579-4581.
- [294] Rodger, A., Marrington, R., Roper, D., Windsor, S., Circular dichroism spectroscopy for the study of protein-ligand interactions. *Methods in molecular biology (Clifton, N.J.)* 2005, 305, 343-364.
- [295] K. Nakanishi, N. B., R.W. Woody, Circular Dichroism, Principles and Applications, VCH, Weinheim, New York, Cambridge, ISBN 1-56081-618-X, 1994. *Berichte der Bunsengesellschaft für physikalische Chemie* 1995, 99, 230-230.
- [296] Bulheller, B. M., Rodger, A., Hirst, J. D., Circular and linear dichroism of proteins. *Phys Chem Chem Phys* 2007, 9, 2020-2035.
- [297] Geninatti Crich, S., Cadenazzi, M., Lanzardo, S., Conti, L., *et al.*, Targeting ferritin receptors for the selective delivery of imaging and therapeutic agents to breast cancer cells. *Nanoscale* 2015, 7, 6527-6533.
- [298] Li, X., Zhang, Y., Chen, H., Sun, J., Feng, F., Protein Nanocages for Delivery and Release of Luminescent Ruthenium(II) Polypyridyl Complexes. *ACS Appl Mater Interfaces* 2016, 8, 22756-22761.
- [299] Kim, J. H., Park, J. M., Roh, Y. J., Kim, I. W., *et al.*, Enhanced efficacy of photodynamic therapy by inhibiting ABCG2 in colon cancers. *BMC Cancer* 2015, 15, 504.
- [300] Matsubara, T., Kusuzaki, K., Matsumine, A., Shintani, K., *et al.*, Acridine orange used for photodynamic therapy accumulates in malignant musculoskeletal tumors depending on pH gradient. *Anticancer research* 2006, 26, 187-193.
- [301] Stock, A. M., Robinson, V. L., Goudreau, P. N., Two-component signal transduction. *Annu Rev Biochem* 2000, 69, 183-215.
- [302] Capra, E. J., Laub, M. T., Evolution of two-component signal transduction systems. *Annu Rev Microbiol* 2012, 66, 325-347.
- [303] Koretke, K. K., Lupas, A. N., Warren, P. V., Rosenberg, M., Brown, J. R., Evolution of Two-Component Signal Transduction. *Molecular Biology and Evolution* 2000, 17, 1956-1970.

- [304] Madan Babu, M., Evolution of transcription factors and the gene regulatory network in *Escherichia coli*. *Nucleic Acids Research* 2003, *31*, 1234-1244.
- [305] Jin, T., Inouye, M., Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1. *Journal of molecular biology* 1993, *232*, 484-492.
- [306] Yang, Y., Inouye, M., Requirement of both kinase and phosphatase activities of an *Escherichia coli* receptor (Taz1) for ligand-dependent signal transduction. *Journal of molecular biology* 1993, *231*, 335-342.
- [307] Alex, L. A., Simon, M. I., Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. *Trends in Genetics* 1994, *10*, 133-138.
- [308] Parkinson, J. S., Kofoed, E. C., Communication modules in bacterial signaling proteins. *Annu Rev Genet* 1992, *26*, 71-112.
- [309] Galperin, M. Y., A census of membrane-bound and intracellular signal transduction proteins in bacteria: bacterial IQ, extroverts and introverts. *BMC Microbiol* 2005, *5*, 35.
- [310] Galperin, M. Y., Nikolskaya, A. N., Koonin, E. V., Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiology Letters* 2001, *203*, 11.
- [311] Moglich, A., Ayers, R. A., Moffat, K., Structure and signaling mechanism of Per-ARNT-Sim domains. *Structure* 2009, *17*, 1282-1294.
- [312] Parkinson, J. S., Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. *Annu Rev Microbiol* 2010, *64*, 101-122.
- [313] Surette, M. G., Levit, M., Liu, Y., Lukat, G., *et al.*, Dimerization Is Required for the Activity of the Protein Histidine Kinase CheA That Mediates Signal Transduction in Bacterial Chemotaxis. *Journal of Biological Chemistry* 1996, *271*, 939-945.
- [314] Pan, S. Q., Charles, T., Jin, S., Wu, Z. L., Nester, E. W., Preformed dimeric state of the sensor protein VirA is involved in plant--*Agrobacterium* signal transduction. *Proceedings of the National Academy of Sciences* 1993, *90*, 9939-9943.
- [315] Yang, Y., Park, H., Inouye, M., Ligand binding induces an asymmetrical transmembrane signal through a receptor dimer. *Journal of molecular biology* 1993, *232*, 493-498.
- [316] Wolfe, A. J., Stewart, R. C., The short form of the CheA protein restores kinase activity and chemotactic ability to kinase-deficient mutants. *Proceedings of the National Academy of Sciences* 1993, *90*, 1518-1522.
- [317] Ninfa, E. G., Atkinson, M. R., Kamberov, E. S., Ninfa, A. J., Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): trans-phosphorylation between subunits. *Journal of Bacteriology* 1993, *175*, 7024-7032.
- [318] Swanson, R. V., Bourret, R. B., Simon, M. I., Intermolecular complementation of the kinase activity of CheA. *Molecular Microbiology* 1993, *8*, 435-441.
- [319] Burbulys, D., Trach, K. A., Hoch, J. A., Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* 1991, *64*, 545-552.
- [320] Cock, P. J., Whitworth, D. E., Evolution of prokaryotic two-component system signaling pathways: gene fusions and fissions. *Mol Biol Evol* 2007, *24*, 2355-2357.

- [321] Egger, L. A., Park, H., Inouye, M., Signal transduction via the histidyl-aspartyl phosphorelay. *Genes to cells : devoted to molecular & cellular mechanisms* 1997, 2, 167-184.
- [322] Forst, S. A., Roberts, D. L., Signal transduction by the EnvZ-OmpR phosphotransfer system in bacteria. *Res Microbiol* 1994, 145, 363-373.
- [323] Pratt, L. A., Silhavy, T. J., Porin Regulon of Escherichia coli. 1995, 105-127.
- [324] Aiba, H., Nakasai, F., Mizushima, S., Mizuno, T., Evidence for the physiological importance of the phosphotransfer between the two regulatory components, EnvZ and OmpR, in osmoregulation in Escherichia coli. *The Journal of biological chemistry* 1989, 264, 14090-14094.
- [325] Inouye, M., Dutta, R., Zhu, Y., Regulation of Porins in Escherichia coli by the Osmosensing Histidine Kinase/Phosphatase EnvZ. 2003, 25-46.
- [326] Forst, S., Inouye, M., Environmentally regulated gene expression for membrane proteins in Escherichia coli. *Annu Rev Cell Biol* 1988, 4, 21-42.
- [327] Igo, M. M., Slauch, J. M., Silhavy, T. J., Signal transduction in bacteria: kinases that control gene expression. *New Biol* 1990, 2, 5-9.
- [328] Igo, M. M., *EnvZ, a transmembrane environmental sensor of Escherichia coli K-12, is phosphorylated in vitro*, 1988.
- [329] Roberts, D. L., Bennett, D. W., Forst, S. A., Identification of the site of phosphorylation on the osmosensor, EnvZ, of Escherichia coli. *The Journal of biological chemistry* 1994, 269, 8728-8733.
- [330] Delgado, J., Forst, S., Harlocker, S., Inouye, M., Identification of a phosphorylation site and functional analysis of conserved aspartic acid residues of OmpR, a transcriptional activator for ompF and ompC in Escherichia coli. *Mol Microbiol* 1993, 10, 1037-1047.
- [331] Igo, M. M., Ninfa, A. J., Stock, J. B., Silhavy, T. J., Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. *Genes Dev* 1989, 3, 1725-1734.
- [332] Yoshida, T., Phadtare, S., Inouye, M., The design and development of Tar-EnvZ chimeric receptors. *Methods in enzymology* 2007, 423, 166-183.
- [333] Smith, E. F., Townsend, C. O., A Plant Tumor of Bacterial Origin. *Science* 1907, 25, 671-673.
- [334] Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P. J. J., *et al.*, The Bases of Crown Gall Tumorigenesis. *Journal of Bacteriology* 2000, 182, 3885-3895.
- [335] Tzfira, T., Citovsky, V., Partners-in-infection: host proteins involved in the transformation of plant cells by Agrobacterium. *Trends in Cell Biology* 2002, 12, 121-129.
- [336] Lee, Y. W., Jin, S., Sim, W. S., Nester, E. W., Genetic evidence for direct sensing of phenolic compounds by the VirA protein of Agrobacterium tumefaciens. *Proceedings of the National Academy of Sciences* 1995, 92, 12245-12249.
- [337] Jin, S., Roitsch, T., Ankenbauer, R. G., Gordon, M. P., Nester, E. W., The VirA protein of Agrobacterium tumefaciens is autophosphorylated and is essential for vir gene regulation. *Journal of Bacteriology* 1990, 172, 525-530.
- [338] Winans, S. C., Mantis, N. J., Chen, C.-Y., Chang, C.-H., Dong Cho, H., Host recognition by the VirA, VirG two-component regulatory proteins of Agrobacterium tumefaciens. *Research in Microbiology* 1994, 145, 461-473.

- [339] Sheng, J., Citovsky, V., Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. *The Plant Cell* 1996, 8, 1699-1710.
- [340] Jin, S. G., Prusti, R. K., Roitsch, T., Ankenbauer, R. G., Nester, E. W., Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. *J Bacteriol* 1990, 172, 4945-4950.
- [341] Mukhopadhyay, A., Gao, R., Lynn, D. G., Integrating Input from Multiple Signals: The VirA/VirG Two-Component System of *Agrobacterium tumefaciens*. *ChemBioChem* 2004, 5, 1535-1542.
- [342] Kiel, C., Yus, E., Serrano, L., Engineering signal transduction pathways. *Cell* 2010, 140, 33-47.
- [343] Becskei, A., Seraphin, B., Serrano, L., Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J* 2001, 20, 2528-2535.
- [344] Becskei, A., Serrano, L., Engineering stability in gene networks by autoregulation. *Nature* 2000, 405, 590-593.
- [345] Fung, E., Wong, W. W., Suen, J. K., Bulter, T., *et al.*, A synthetic gene-metabolic oscillator. *Nature* 2005, 435, 118-122.
- [346] Swinburne, I. A., Miguez, D. G., Landgraf, D., Silver, P. A., Intron length increases oscillatory periods of gene expression in animal cells. *Genes Dev* 2008, 22, 2342-2346.
- [347] Bulter, T., Lee, S. G., Wong, W. W., Fung, E., *et al.*, Design of artificial cell-cell communication using gene and metabolic networks. *Proceedings of the National Academy of Sciences* 2004, 101, 2299-2304.
- [348] Balagadde, F. K., Song, H., Ozaki, J., Collins, C. H., *et al.*, A synthetic *Escherichia coli* predator-prey ecosystem. *Mol Syst Biol* 2008, 4, 187.
- [349] Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., Keasling, J. D., Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol* 2003, 21, 796-802.
- [350] Friedland, A. E., Lu, T. K., Wang, X., Shi, D., *et al.*, Synthetic Gene Networks That Count. *Science* 2009, 324, 1199-1202.
- [351] Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., Weiss, R., A synthetic multicellular system for programmed pattern formation. *Nature* 2005, 434, 1130-1134.
- [352] Isalan, M., Santori, M. I., Gonzalez, C., Serrano, L., Localized transfection on arrays of magnetic beads coated with PCR products. *Nat Methods* 2005, 2, 113-118.
- [353] Gossen, M., Bujard, H., Studying gene function in eukaryotes by conditional gene inactivation. *Annu Rev Genet* 2002, 36, 153-173.
- [354] Deans, T. L., Cantor, C. R., Collins, J. J., A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells. *Cell* 2007, 130, 363-372.
- [355] Tigges, M., Marquez-Lago, T. T., Stelling, J., Fussenegger, M., A tunable synthetic mammalian oscillator. *Nature* 2009, 457, 309-312.
- [356] Pryciak, P. M., Designing new cellular signaling pathways. *Chem Biol* 2009, 16, 249-254.

- [357] Teruel, M. N., Meyer, T., Translocation and Reversible Localization of Signaling Proteins. *Cell* 2000, *103*, 181-184.
- [358] Jordan, J. D., Landau, E. M., Iyengar, R., Signaling Networks. *Cell* 2000, *103*, 193-200.
- [359] Aravind, L., Anantharaman, V., Iyer, L. M., Evolutionary connections between bacterial and eukaryotic signaling systems: a genomic perspective. *Current Opinion in Microbiology* 2003, *6*, 490-497.
- [360] Galperin, M. Y., Gomelsky, M., Bacterial signal transduction modules: from genomics to biology. *ASM News* 2005, *71*, 326-333.
- [361] Galperin, M. Y., Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* 2004, *6*, 552-567.
- [362] Finlay, B. B., Falkow, S., Common themes in microbial pathogenicity revisited. *Microbiology and molecular biology reviews : MMBR* 1997, *61*, 136-169.
- [363] Sansonetti, P. J., Tran Van Nhieu, G., Egile, C., Rupture of the intestinal epithelial barrier and mucosal invasion by *Shigella flexneri*. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1999, *28*, 466-475.
- [364] Isberg, R. R., Leong, J. M., Multiple beta 1 chain integrins are receptors for invasins, a protein that promotes bacterial penetration into mammalian cells. *Cell* 1990, *60*, 861-871.
- [365] Leong, J. M., Fournier, R. S., Isberg, R. R., Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. *Embo j* 1990, *9*, 1979-1989.
- [366] Leahy, D. J., Aukhil, I., Erickson, H. P., 2.0 Å crystal structure of a four-domain segment of human fibronectin encompassing the RGD loop and synergy region. *Cell* 1996, *84*, 155-164.
- [367] Rankin, S., Isberg, R. R., Leong, J. M., The integrin-binding domain of invasin is sufficient to allow bacterial entry into mammalian cells. *Infection and immunity* 1992, *60*, 3909-3912.
- [368] Tran Van Nhieu, G., Isberg, R. R., Bacterial internalization mediated by beta 1 chain integrins is determined by ligand affinity and receptor density. *Embo j* 1993, *12*, 1887-1895.
- [369] Lee, T. S., Krupa, R. A., Zhang, F., Hajimorad, M., *et al.*, BglBrick vectors and datasheets: A synthetic biology platform for gene expression. *J Biol Eng* 2011, *5*, 12.
- [370] Anderson, J. C., Dueber, J. E., Leguia, M., Wu, G. C., *et al.*, BglBricks: A flexible standard for biological part assembly. *J Biol Eng* 2010, *4*, 1.
- [371] Subashchandrabose, S., Smith, S. N., Spurbeck, R. R., Kole, M. M., Mobley, H. L., Genome-wide detection of fitness genes in uropathogenic *Escherichia coli* during systemic infection. *PLoS Pathog* 2013, *9*, e1003788.
- [372] Isberg, R. R., Voorhis, D. L., Falkow, S., Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* 1987, *50*, 769-778.
- [373] Isberg, R. R., Barnes, P., Subversion of integrins by enteropathogenic *Yersinia*. *Journal of Cell Science* 2001, *114*, 21.

- [374] Isberg, R. R., Hamburger, Z., Dersch, P., Signaling and invasin-promoted uptake via integrin receptors. *Microbes and Infection* 2000, 2, 793-801.
- [375] Fajac, I., Grosse, S., Collombet, J. M., Thevenot, G., *et al.*, Recombinant *Escherichia coli* as a gene delivery vector into airway epithelial cells. *J Control Release* 2004, 97, 371-381.
- [376] Campos, E. A., Stockler Pinto, D. V. B., Oliveira, J. I. S. d., Mattos, E. D. C., Dutra, R. D. C. L., Synthesis, Characterization and Applications of Iron Oxide Nanoparticles - a Short Review. *Journal of Aerospace Technology and Management* 2015, 7, 267-276.
- [377] Lonn, P., Kacsinta, A. D., Cui, X. S., Hamil, A. S., *et al.*, Enhancing Endosomal Escape for Intracellular Delivery of Macromolecular Biologic Therapeutics. *Sci Rep* 2016, 6, 32301.
- [378] Kaiser, C. R., Flenniken, M. L., Gillitzer, E., Harmsen, A. L., *et al.*, Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance in vivo. *International Journal of Nanomedicine* 2007, 2, 715-733.
- [379] Yang, Y., Neef, T., Mittelholzer, C., Garcia Garayoa, E., *et al.*, The biodistribution of self-assembling protein nanoparticles shows they are promising vaccine platforms. *Journal of Nanobiotechnology* 2013, 11, 36-36.
- [380] Riedl, A., Schleiderer, M., Pudelko, K., Stadler, M., *et al.*, Comparison of cancer cells in 2D vs 3D culture reveals differences in AKT-mTOR-S6K signaling and drug responses. *J Cell Sci* 2017, 130, 203-218.
- [381] Zanoni, M., Piccinini, F., Arienti, C., Zamagni, A., *et al.*, 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. *Sci Rep* 2016, 6, 19103.
- [382] Fang, Y., Eglén, R. M., Three-Dimensional Cell Cultures in Drug Discovery and Development. *SLAS Discov* 2017, 22, 456-472.
- [383] Short, S. P., Costacurta, P. W., Williams, C. S., Using 3D Organoid Cultures to Model Intestinal Physiology and Colorectal Cancer. *Curr Colorectal Cancer Rep* 2017, 13, 183-191.
- [384] Young, M., Reed, K. R., Organoids as a Model for Colorectal Cancer. *Curr Colorectal Cancer Rep* 2016, 12, 281-287.
- [385] Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., *et al.*, Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 2009, 6, 343-345.

Appendix I

1. Sequence of *FieF* along with 50bp upstream and 50bp downstream.

>gi|49175990:4104442-4105444 Escherichia coli str. K-12 substr. MG1655, complete genome

CAGATGATTTGCTTCCGTTATACTAGCGTCAGTTGATAGCGGGAGTATT
TATGAATCAATCTTATGGACGGCTGGTCAGTCGGGCGGCGATTGCTGCG
ACGGCGATGGCTTCGCTGCTATTGCTGATTAATAATTTTGCATGGTGGT
ATACCGGGTCGGTGAGTATTCTCGCCGCGCTGGTGGATTCTGCTGGTGGA
TATCGGCGCGCTCGTTGACGAATTTATTGGTGGTGCGATATTCCCTGCAA
CCTGCCGACGATAATCACTCGTTTGGTCACGGTAAAGCTGAGTCCCTCG
CGGCGCTGGCGCAAAGTATGTTTATCTCCGGTTCGGCACTATTCCTGTTT
TTGACGGGTATTCAACATCTGATATCTCCAACACCGATGACAGATCCAG
GCGTCGGGGTTATCGTGACAATTGTGGCGCTAATTTGTACGATTATCCT
TGTCTCGTTTCAGCGTTGGGTGGTGCGGCGGACGCAAAGCCAGGCGGT
GCGGGCTGATATGCTACATTACCAGTCTGATGTTATGATGAACGGCGCA
ATTCTGCTGGCGCTGGGGTTGTCCTGGTACGGCTGGCATCGCGCCGATG
CTCTGTTTGCATTGGGAATCGGCATCTATATTTTATATAGCG
CGTTACGCATGGGATATGAGGCGGTACAGTCATTACTGGATCGCGCATT
GCCTGATGAGGAACGGCAAGAAATTATTGATATCGTGACTTCCTGGCCG
GGTGTTAGCGGCGCTCACGATCTTCGCACGCGGCAGTCAGGGCCGACC
CGCTTTATTACGATTCATTTGGAAATGGAAGACTCTCTGCCTTTGGTTCA
GGCACATATGGTGGCGGATCAGGTAGAGCAGGCTATTTTACGGCGTTTT
CCGGGATCGGATGTAATTATCCATCAGGACCCCTGTTCCGTCGTACCCA
GGGAGGGTAAACGGTCTATGCTTTTCATAATCAGTATAAAAGAGAGCCA
GACCCGCATTTTGTGTATAAAATACCGCCAT

2. Transformation into Bacterial Host

Transformation was performed by keeping the competent cells on ice for 10 minutes. Plasmid or linear DNA (5µl) was added to the competent cells and incubated on ice for 15 minutes. Then a 45 seconds heat shock at 42 °C was given followed by incubation on ice for 2 minutes. LB (500 µl) was added to the cells and incubated in a shaker for 1 hour at 37 °C and 200rpm. Cells were plated on LB plates with suitable antibiotics and incubated for 16 hours at 37°C.

3. DNA Gel Electrophoresis

Gel electrophoresis was performed in 1.5% agarose gel in TAE buffer. Agarose was dissolved in TAE buffer (1X) by heating and was then cooled to about 50 °C. 1/10,000 volume of 10,000X gel star was added to the solution and gently mixed by shaking. The set was fixed on the gel caster and agarose was poured gently and allowed to solidify at room temperature for 30 minutes. The comb was removed gently and the gel caster tray was placed in the buffer tank and covered completely with TAE buffer. The DNA products were mixed with the gel loading dye and loaded carefully along with 1Kb ladder in a separate well. Electrophoresis was carried out at 100 volts for 30 minutes. The gel was view and photographed in a Syngene G:BOX gel doc system.

Appendix II

1. Hydrophobic interaction chromatogram of AfFtnAA

Figure 10.1: Hydrophobic interaction chromatograms for AfFtnWT with inset showing the SDS gel for cell lysate (L), supernatant after heat treatment (S), pellet after heat treatment (P), and fractions A4, 5 and A6. 20 kDa band corresponds to AfFtnWT monomers.

Appendix III

Acridine Orange Standard Plot

Figure 11.1: Standard absorbance plot of AO in 0 to 50 μ M range

Appendix IV

Standard restriction digestion protocols used for assembling the parts

Standard restriction digestion and ligation protocols were used to clone two fragments together which follow the RFC 21 standard biobrick assembly method [370]. This standard uses the enzymes EcoRI and BglII as the prefix and BamHI and XhoI as the suffix.

Digestion: 1 µg of the DNA (both vector and insert) were digested with suitable restriction enzymes for 15 mins at 37 °C. 1ul of calf intestinal alkaline phosphatase was added to the vector and the reaction was continued for further 15 mins. Agarose gel electrophoresis was used to separate the DNA bands which were gel extracted using Qiagen gel extraction kit.

Ligation: Ligation was carried out with a vector to insert molar ratio of 1:5. The ligation reaction was performed at room temperature for 30 mins using NEB T4 DNA ligase enzyme and was followed by transformation. Standard transformation protocols were used for transformation, except plates were left at room temperature for 24 to 48 hours for the appearance of colonies.

Gibson Assembly: Gibson assembly was used to assemble multiple parts together in one step [385]. PCR fragments were generated with 30 bp overhangs and treated with DpnI enzyme to remove the backbone plasmid DNA and purified in warm water as eluent. Concentration of the purified products were measure using Nanodrop. Fragments were added along with water to make up volume to 5 µl and 5 µl of HiFi DNA Assembly mix (New England Biolabs) was added and the reaction

was performed at 50 °C for 15 minutes to 45 minutes (based on manufacturers protocol). 4 µl of the assembled product was transformed into chemically competent *E. coli* TOP10. Following are the details of the Gibson assemblies used in the study.

Construct 1

Assembly Preview

Assembly Primers

Primer	Bases	T _m whole (°C)	T _i
J23101 - RBS FWD (1)	ttaccagaggcgccccagctggcaattccgagcgttattgacaaacttgacggcctc	75.7	
J23101 - RBS REV (1)	GCTGCGTGGTGAAAAACGCAGGCGGCGCATttccctctttccagaaccGCTAGCAT	76.7	
pBAD-InvEnvZ FWD	TATGCTAGCggatctgaaagaggggaaaATGCGCCGCCGCGT11111CACCACGCAGC	76.7	
pBAD-InvEnvZ REV	gcctggagatcctactcgagtttgatccTCA11AACCCTC111CG1CGTGCC11GCGC	74.2	
J23101 - RBS FWD (2)	GCGCAAGGCACGACGAAAGAGGGTTAATGaggaiccaaactcgagaaaggaiccccgcc	74.2	
J23101 - RBS REV (2)	gatgtagccgtcaagttgtcataagacgtcggcattgccagctggggcgccctcggiaa	75.7	

Construct 2

Assembly Preview

Assembly Primers

Primer	Bases	T_m whole (°C)	T_i
J23101 - RBS FWD (1)	ttaccagagggcgccccagctggcaattccgacgcttcatgacaacttgacggcicacac	75.7	
J23101 - RBS REV (1)	TTGCCGAGACGGTGAATACCTTCCATTCATitccccctttccagatccGCTAGCA7A	72.2	
pBAD - InvVirA FWD	TATGCTAGCggatctggaagaggggaaaATGAA7GGAAGGTAT7CACCG7CTCGGCAA	72.2	
pBAD - InvVirA REV	gcctggagatccttactcgagtttggatccCTAGGC777GA7C77GG7ACGAA7CGCG7A	72.6	
J23101 - RBS FWD (2)	TACGCGATTCTGACCAAGATCAAAGCCTAGggatccaaacgcgagaaaggatctccaggc	72.6	
J23101 - RBS REV (2)	gatgtagccgtcaagttgtcataagacgtcggattgccagciggggcgccctcggiaa	75.7	

Construct 3

Assembly Preview

Assembly Primers

Primer	Bases	T _m ^{whole} (°C)	T _i
J23101 - RBS FWD (1)	ttaccagagggcgccccagctggcaattccgacgicctatgacaacttgacggcicacac	75.7	
J23101 - RBS REV (1)	CTGACGACTTGGGGAATAGCGACCGTTCATttccctcttttcagatccGCTAGCATA	73.1	
pBAD-Invasin FWD	TATGCTAGCgcatctgaaaagaggggaaaATGAACGGTCGCTAT1CCCCAAGTCG7CAG	73.1	
pBAD-Invasin REV	gcctggagatccttactcgagittgatccT1AGGC777GATC77CG7ACGAA7GGCATA	71.2	
J23101 - RBS FWD (2)	TATGCCATTCTGTACGAAGATCAAAGCCTAAggatccaactcgagtaaggatciccaggc	71.2	
J23101 - RBS REV (2)	gatgtagccgtcaagttgtcataagacgtcggaaatgccagciggggcgccctciggtaa	75.7	

Construct 4

Assembly Preview

Assembly Primers

Primer	Bases	T _m ^{whole} (°C)	T _m ^{ar} (°C)
J23101 - InvEnvZ FWD (1)	gctgaaggtcgtcactccaccggtgcttaaggatc caaa ctcgag aa gga at ctccaggc	74.5	63
J23101 - InvEnvZ REV (1)	gatgtagccgtcaagttgtcataagacgtc gga aattgc ac gicggggcgcctctcggtaa	75.7	70
J23101 - InvEnvZ FWD (2)	ttaccagaggcgccccagctggcaattccgacgic aat gacaacttga cg gcacatccttcacitctttcac	75.5	66
J23101 - InvEnvZ REV (2)	gaactctttgataacgtcttcgtactcgc ACCC TCTTTCTGTCGTGCC TT GC GC GC GC GT	77.0	73
pBAD - RFP FWD	ACGCGCGCGCAAGGCACGACGAAAGAGGGT gc gagtagcgaagacg aat caagagttc	77.0	61
pBAD - RFP REV	gcctggagatccttactcgagtttgatcct ta agcac cg gtggagigacgaccttcagc	74.5	66