Development of New Series of Protein Based MRI Contrast Agents: ProCA1 Variants and Humanized ProCA32

Anvi N. Patel

Georgia State University, apatel117@gsu.edu

Follow this and additional works at: https://scholarworks.gsu.edu/chemistry_theses

Recommended Citation
doi: https://doi.org/10.57709/7029162
DEVELOPMENT OF NEW SERIES OF PROTEIN BASED MRI CONTRAST AGENTS:
PROCA1 VARIANTS AND HUMANIZED PROCA32

by

ANVI PATEL

Under the Direction of Jenny J. Yang, Ph.D.

ABSTRACT

MRI is a noninvasive technique used for disease diagnosis. However, recent clinically used MRI contrast agents exhibit low relaxivity, high metal toxicity due to low dose efficiency and inability to target specific diseased cells. To address the pressing unmet clinical needs, we developed a novel class of protein-based contrast agent, with improved sensitivity, high relaxivity with better metal selectivity, stability and low toxicity[1]. A GRPR-targeted ProCA1 variants designed to selectively target prostate cancer via molecular imaging, were successfully expressed in bacterial expression system and tagless purification, showed about 12 fold higher relaxivities as compared to Gd-DTPA. hProCA32 demonstrated strong Gd^{3+} binding affinity with $K_d$ of $10^{-22}$ M and strong selectivity for Gd^{3+} over Ca^{2+} and Zn^{2+}, high $r_1$ (28 mM$^{-1}$s$^{-1}$) and $r_2$ (35 mM$^{-1}$s$^{-1}$) and PEGylated-hProCA32 showed excellent in vitro serum stability for 12 days. These studies demonstrate stronger translational potentials of ProCAs for human applications.

INDEX WORDS: Magnetic Resonance Imaging, Contrast agents, Gadolinium, Relaxivity, Metal binding, PEGylation,
DEVELOPMENT OF NEW SERIES OF PROTEIN BASED MRI CONTRAST AGENTS:

PROCA1 VARIANTS AND HUMANIZED PROCA32

by

ANVI PATEL

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2015
DEVELOPMENT OF NEW SERIES OF PROTEIN BASED MRI CONTRAST AGENTS:
PROCA1 VARIANTS AND HUMANIZED PROCA32

by

ANVI PATEL

Committee Chair: Jenny J. Yang
Committee: Robert Wohlhueter
Siming Wang
Ming Luo

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2015
DEDICATION

I would like to a moment to express my gratitude to my parents Nimish Patel and Alpana Patel, my brother Harshal Patel, my grandparents, other family members and my best friend Harleen Nayyar for their continuous support and love. I am grateful to my parents for all the sacrifices and hard work they have put in giving me a bright future and supporting me throughout my education. My parents taught me to believe in myself, which helped me to have confidence and earn this degree. I would specially like to thank my grandparents who always cheered me up and taught me to be optimistic in every difficult situation and never give up. I would also like to thank my brother who cheered me and showed his dedicated and unwavering support in fulfilling my goal. I would also like to thank my best friend Harleen, who showed endless patience, and her unconditional emotional support. I am grateful for having such encouraging and loving people who made it possible for me to strive for the best. I would like to dedicate this work to all the people who played a major role in making me the person who I am today.
ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my advisor, Dr. Jenny J. Yang. I am grateful to her for giving me such wonderful opportunity to expand my knowledge and experience. Her motivation, passion for science and excellence in creative research ideas has inspired me to work hard and except all the challenges that came along. I am honored to have worked under her guidance and have acquired the foundation during the critical years of my academic career. Her high standards for quality of work encouraged me to work even harder and put extra effort. Her broad array of knowledge in the field of biochemistry and protein chemistry has given me a direction to choose a career path in these fields. I would like to thank Dr. Fan Pu as senior mentor, she gave me initial training and expanded my knowledge in protein expression and purification with solid base. Next, I would like to thank Dr. Shenghui Xue for his detailed and rigorous training for this project and teaching the necessary skill that helped my critical analysis of data. I greatly appreciate both Dr. Fan and Dr. Shenghui for their undivided attention and willingness to help me in every challenge I faced. Without their suggestion and detailed help, I could not have finished my thesis. My heartfelt thanks to Dr. JinjuanQiao, and Rose Auguste for being patience with me and being eager to answer my countless questions. I am grateful for all the support and help from members of MRI project Shanshan Tan, Corrie Purser and Chaky Muankaew, I would like to thank other lab member especially Dr. Chen Zhang and Juan Zou for their suggestions and help in developing all the mutants. I would also like to thank other lab members Dr. Yusheng Jiang, Dr. Yanyi Chen, Dr.You Zuo, Xueyun Liu, Florence Reddish, Li Zhang, Mani Salarian, Rakshya Gorkhali, Cassandra Miller and Fantasha Goolsby for their suggestions and patience during the group discussion. Lastly, I would like to express my heartfelt gratitude to my committee member Dr.
Robert Wolhueter for his support and critical analysis of my thesis and Dr. Siming Wang for her guidance and time invested to support my research. I sincerely thank Dr. Ming Luo for accepting my invitation at late notice and grateful for detailed analysis and assistance for supporting my research.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... V

LIST OF FIGURES .................................................................................................................. XI

LIST OF TABLES ................................................................................................................... XVII

LIST OF ABBREVIATIONS .............................................................................................. XVIII

1. INTRODUCTION.................................................................................................................. 1

1.1. Cancer ............................................................................................................................. 1

1.2. Clinical Imaging Modalities ......................................................................................... 1

1.3. Cancer Biomarkers and Targeted Molecular Imaging ................................................. 3

1.4. Magnetic Resonance Imaging (MRI) ........................................................................... 8

1.5. Contrast Agents in MRI ............................................................................................... 13

1.5.1. Current MRI Contrast Agents and their limitations ................................................. 14

1.5.2. Criteria for designing Contrast Agents .................................................................. 18

1.6. Designing Protein-based MRI contrast agents – a novel approach ......................... 19

1.6.1. ProCA1 ................................................................................................................... 22

1.6.2. Grafting approach on ProCA1 for targeting GRPR ................................................. 26

1.6.3. ProCA32 .............................................................................................................. 29

1.7. Additional challenges in developing a Protein Based MRI Contrast Agents for clinical applications .................................................................................................................... 35

1.7.1. Approaches to reduce Immunogenicity ................................................................. 35

1.8. Objective of this Thesis ............................................................................................. 36

2. MATERIALS AND METHODS ....................................................................................... 39
2.1. Expression of ProCA1 and its variant ................................................................. 39
   2.1.1. Cloning ........................................................................................................ 39
   2.1.2. Transformation .......................................................................................... 40
   2.1.3. Inoculation ............................................................................................... 41
   2.1.4. Expression ............................................................................................... 41
2.2. Tagless purification of ProCA1 and its variants .................................................... 43
   2.2.1. Sonication and cell disruptor .................................................................... 43
   2.2.2. Purification by FPLC: anion exchange HiTrap Q Column ....................... 44
2.3. Expression of humanized ProCA32 (hProCA32) .................................................. 47
   2.3.1. Expression of \(^{15}\)N Labeled hCA32 ......................................................... 48
2.4. Tagless Purification of humanized ProCA32 (hProCA32) .................................... 49
   2.4.1. Sonication and Boiling ............................................................................ 49
   2.4.2. Nucleic Acid Precipitation ...................................................................... 49
2.5. Molecular Cloning ............................................................................................. 50
   2.5.1. Site directed Mutagenesis for humanized ProCA32.Cys ............................ 50
   2.5.2. Plasmid reconstruction of pRSET-CD20 .................................................. 51
2.6. Expression and purification of hCa32.cys ............................................................ 51
2.7. Concentration Determination using Ultraviolet-visible absorbance spectra ........... 51
2.8. Western Blotting .............................................................................................. 52
2.9. Relaxivity measurements ................................................................................... 53
2.10. Terbium Titration using Metal-Chelator Buffer System ..................................... 54
2.11. Determining Metal Binding Affinity: Gd\(^{3+}\), Zn\(^{2+}\), Ca\(^{2+}\), Lu\(^{3+}\) .................. 55
2.12. PEGylation ..................................................................................................... 57
2.12.1. Lys-PEG .............................................................................................................. 58

2.12.2. Cys- PEG ............................................................................................................. 58

2.13. Serum Stability ........................................................................................................... 59

3. DEVELOPMENT OF RAT PROCA1 AND ITS ENGINEERED VARIANTS VIA
   GRAFTING APPROACHES .......................................................................................... 60

   3.1. Introduction ............................................................................................................... 60

   3.1. Results ......................................................................................................................... 61

   3.1.1. Expression of ProCA1 and its variants .............................................................. 61

   3.1.2. Tagless purification by denaturing and refolding method .............................. 68

   3.1.3. Determination of Relaxivity ............................................................................. 79

   3.1.4. Fluo-Zn competition assay ............................................................................. 83

   3.2. Conclusions .............................................................................................................. 85

4. OPTIMIZING THE EXPRESSION AND PURIFICATION OF HUMANIZED
   PROCA32 AND ITS MUTANT .................................................................................. 87

   4.1. Introduction ............................................................................................................... 87

   4.2. Results ......................................................................................................................... 88

   4.2.1. Developing the Expression and Purification of hCA32 based on previously
          optimized protocol of rat ProCA32. ........................................................................ 88

   4.2.2. Optimizing the expression and purification for $^{15}$N labeled hCA32 .......... 104

   4.2.3. Molecular cloning of hCA32.cys ................................................................. 110

   4.2.4. Expression and purification of hProCA32.cys ................................................ 115

   4.2.5. Purification of rat ProCa32 expressed using fermenter ................................. 120

   4.3. Conclusions .............................................................................................................. 125
5. RELAXIVITY, METAL BINDING, SELECTIVITY AND STABILITY

OF ENGINEERED HPROCA32

5.1. Introduction

5.2. Results

5.2.1. Site specific Lysine and Cysteine PEGylation

5.2.2. Relaxivity measurements

5.2.3. Equilibrium Calcium titration

5.2.4. Determining metal binding affinity of hProCA32 using Tb³⁺ EGTA/DTPA buffer system

5.2.5. Determining the affinity of hProCA32 to Gd³⁺ using various fluorescence assays

5.2.6. Determining Zn²⁺ binding affinity of hProCA32 using a FluoZin-1 competition assay

5.2.7. Serum stability of hProCA32

5.2.8. Magnetic Resonance imaging of cysteine PEGylated hProCA32 and Future Directions

5.3. Conclusion and Future directions

6. OVER ALL CONCLUSION AND SIGNIFICANCE OF THIS WORK

REFERENCE
LIST OF FIGURES

Figure 1.1 Illustration of various biomarkers expressed under cancerous environment. ..................5
Figure 1.2 Biomarkers that are recognized by FDA considering its sensitivity and specificity in molecular imaging. Adapted from the reference with permission [7] .............................................. 6
Figure 1.3 Schematic representation of nuclei under magnetic field and their relaxation process. .................................................................................................................................................. 11
Figure 1.4 Diagram depicting the process of (A) R1 relaxivity and (B) R2 relaxivity. ................. 12
Figure 1.5 Commercially available, clinically used and FDA approved MRI contrast agents. Adapted with permission from reference [25] ................................................................. 16
Figure 1.6 List of contrast agents with certain restrictions and dosage approval worldwide. Adapted with permission from reference [28] ................................................................. 17
Figure 1.7 Schematic representation of the factors that can be optimized to improve the properties of protein based contrast agent. ................................................................. 21
Figure 1.8 Model structure of ProCA1 with binding gadolinium binding pocket and its coordination. (Generated by Chimera UCSF) ................................................................. 25
Figure 1.9 The sequence alignment of conserved residues at C-terminal of GRP and Bombesin. Binding affinities of ligands targeting GRPR. Adapted with permission from reference [1] ...... 27
Figure 1.10 Two approached: engineering of targeting sequence via (left) inserting it at C-terminal of ProCA1 (ProCA1.GRPC) and (Right) grafting into the ProCA1 sequence between Gly52 and Gly53 (ProCA1.GRP (52)). Adapted with permission from reference [27] ................. 28
Figure 1.11 Model structure of ProCA1 grafted with G10, B10 and B14 using flexible linker GGSGG (obtained by ITASSER)(Shenghui Xue’s dissertation) [39] .................................................... 29
Figure 1.12 A Cartoon model of rat ProCA32 and MRI images ................................................. 32
Figure 1.13 Protein sequence alignment of hCA32 with rat ProCA32, wild type rat Parvalbumin (ProCA30), wild type human parvalbumin (hCA30). ................................................................. 33
Figure 1.14 Overlay of hCA32 and rat ProCA32 using UCSF Chimera ........................................ 34
Figure 2.1 pET-20b(+) vector for ProCA1 variants overexpression. ............................................ 39
Figure 2.2 Summary of Transformation (top) and Expression procedure (Bottom) ............... 42
Figure 2.3 Summary of Purification Process for ProCA1 and its variants. ............................... 46
Figure 2.4 Map of pET-22b(+) vector for hCA32 variants overexpression. ............................. 47
Figure 3.1 Small scale expression of ProCA1 in various *E.coli* cell strains ......................... 63
Figure 3.2 Large scale Expression of ProCA1 in *E.coli* cell strains using LB medium ........ 66
Figure 3.3 15 % SDS-PAGE of the overexpression of (A) ProCA1.B10, (B) ProCA1.G10 and (C) ProCA1.B14 in Bl21 (DE3) competent cells ................................................................. 67
Figure 3.4 Salt gradient program for the isolation of ProCA1 variants using anion exchange Q column in FPLC ................................................................. 70
Figure 3.5 Tagless Purification of ProCA1 expressed in BL21 (DE3) competent cells by Refolding method ......................................................................................... 71
Figure 3.6 Purification by FPLC of another batch of ProCA1 expressed in BL21 (DE3) competent cells ............................................................................... 72
Figure 3.7 The tagless purification of ProCA1 expressed in Tuner cell strain by refolding method ......................................................................................... 74
Figure 3.8 15 % SDS PAGE depicting the purification steps of ProCA1.G10 .................... 75
Figure 3.9 15 % SDS PAGE depicting the purification steps of ProCA1.B14 expressed in BL21(DE3) cell strain ................................................................. 76
Figure 3.10 15 % SDS-PAGE analysis of the purification process of ProCA1.B10 expressed in BL21(DE3) *E. coli* cell strain. ................................................................. 77

Figure 3.11 Western Blot and 15 % SDS-PAGE of purified ProCA1 variants. ...................... 78

Figure 3.12 Relaxivity measurements of ProCA1 variants. .................................................. 82

Figure 3.13 Determination of Zn$^{2+}$ binding affinity of ProCA1 variants using Fluo-Zn1 dye competition assay. ........................................................................................................ 84

Figure 4.1 Cellular growth curve for the small scale expression of hCA32 in (A) BL21(DE3) (B) Tuner and their corresponding 15 % SDS-PAGE. .................................................. 89

Figure 4.2 The Large scale expression of hCA32 in *E. coli* (A) (B) Cellular growth curve and 15 % SDS PAGE hCA32 expressed in BL21(DE3) respectively and (C)(D) Cellular growth curve and 15% SDS PAGE of hCA32 in BL21(DE3).pLysS ................................................................. 91

Figure 4.3 The purification gel of hCA32 based on Dr.M. Henzl’s protocol modified by Dr. ShenghuiXue for rat ProCA32 ............................................................................................................ 93

Figure 4.4 FPLC salt gradient program for hCA32 purification. .............................................. 95

Figure 4.5 The Uv spectrum of calcium free (dashed line) and calcium loaded (solid line) form of Parvalbumin F102W ........................................................................................................ 97

Figure 4.6 A typical FPLC chromatogram of hProCA32 by anion exchange Q-column separation, the 15 % SDS-PAGE and UV absorbance of each peak eluted for hProCA32 expressed in BL21 (DE3) competent cells ................................................................. 98

Figure 4.7 A typical FPLC chromatogram of hProCA32 by anion exchange Q-column separation, the 15 % SDS-PAGE and UV absorbance of each peak- hProCA32 expressed in BL21(DE3).pLysS ............................................................................................................ 99
Figure 4.8 15% SDS-PAGE gel of hProCA32 (A) expressed in BL21 (DE3) and (B) purification.

Figure 4.9 A typical FPLC chromatogram of hProCA32 purified by anion exchange chromatography and the 15% SDS-PAGE of the fraction corresponding to the chromatogram.

Figure 4.10 UV Spectrum of the protein purified by anion exchange chromatography.

Figure 4.11 Overexpression and purification of hCA32 in M9 minimal medium (A) Western blot (B) 15% SDS PAGE of hCA32 expressed in M9 minimal medium and LB medium. (C) Purification of hCA32 from M9 medium (D) UV spectra of pure hCA32.

Figure 4.12 Over expression of 15N labeled hCA32 using M9 minimal medium.

Figure 4.13 The purification of 15N labeled hCA32 expressed in M9 minimal medium.

Figure 4.14 The purification of 15N labeled hCA32 using FPLC. (A) FPLC chromatogram (B) 15% SDS PAGE (C) Western Blot (D) UV spectra of the fractions eluted by FPLC Q column.

Figure 4.15 The UV spectra of (A) Peak 2 and (B) Peak 3 from FPLC elution after passing them through desalting column.

Figure 4.16 Possible Lysine (Cyan) and Cysteine (Beige) PEGylation sites on hProCA32.

Figure 4.17 Molecular cloning of hCA32.cys by PCR (A) Forward and Reverse Primer design for site directed mutagenesis of hCA32 (B) DNA gel electrophoresis of the plasmid obtained after PCR.

Figure 4.18 DNA and protein sequence alignment of mutated variant hCA32.cys with hCA32.
Figure 4.19 Overexpression of hCA32.cys in *E. coli* (A) Cellular growth curve (B) 15% SDS-PAGE of hCA32.cys expressed in BL21 (DE3).pLysS and LB medium

Figure 4.20 SDS-APGE of purification of hCA32.cys where no reducing agent was added to the sample buffer. However, the protein solution contained 5 mM DTT as a reducing agent.

Figure 4.21 (A) Typical FPCL chromatogram of hCA32.cys purified by Q- column separation, (B) The spectrum of re-chromatography of peak 1 from A(C) the UV absorbance and (D) 15% SDS-APGE gel of each fraction.

Figure 4.22 UV absorbance of concentrated hCA32.cys

Figure 4.23 The purification gel of ProCA32 based on the purification method from Shenghui Xue’s dissertation

Figure 4.24 FPLC Spectrum of anion exchange Q- column

Figure 4.25 UV-Vis Spectra indicates a successful separation of ProCA32 in 10 mM HEPES pH 7.2

Figure 5.1 Iodine stained and coomassie blue stained gels depicting the PEGylation of hCA32.cys

Figure 5.2 The mass spectra of hProCA32.cys before and after PEGylation

Figure 5.3 The measurement of T₁ and T₂ relaxation time for hProCA32 with 100 μM Gd³⁺

Figure 5.4 The relaxivity measurements of hCA32 compared with rat ProCA32 expressed by fermentation

Figure 5.5 Determining the metal binding affinity of hProCA32 using metal chelator buffer system

Figure 5.6 Fluorescence spectra of hProCA32 binding with Tb³⁺ using metal chelator buffer system
Figure 5.7 Determining Tb $^{3+}$ binding affinity of hProCA32 using Tb$^{3+}$-EGTA / DTPA system .................................................................................................................................................... 143

Figure 5.8 Determining binding affinity of hProCA32 for Gadolinium using Fluo-5N competition assay .................................................................................................................................................................................. 145

Figure 5.9 Determining the Gd$^{3+}$ binding affinity of hProCA32 using competition assay with Tb$^{3+}$ ............................................................................................................................................ 147

Figure 5.10 Determining Zinc binding affinity of hCA32 ................................................................................................................................. 149

Figure 5.11 Determining Lutetium binding affinity of hProCA32. $\lambda_{ex}=288$ nm, $\lambda_{em}=546$ nm 10 $\mu$M hProCA32, 20 $\mu$M Tb$^{3+}$ in 10 mM HEPES 150, mM NaCl, pH7.2 .............................................................. 149

Figure 5.12 The serum stability study of hCA32 at 37°C in presence of gadolinium. ................................................................. 151

Figure 5.13 The serum stability study of Cysteine PEGylated -hCA32 at 37°C in presence of gadolinium. ............................................................................................................................................................................... 152

Figure 5.14 The serum stability study of Lysine PEGylated-hProCA32 at 37°C in presence of gadolinium. ............................................................................................................................................................................... 154

Figure 5.15 T1 weighted- Gradient echo sequenced MRI images of kidney and Liver before and after injection of hProCA32.cys-2kDa PEG.cys into the mice via tail vein injection, .......... 156

Figure 5.16 T1 weighted- Fast spin echo sequenced MRI images of Liver before and after injection of hProCA32.cys-2kDa PEG.cys into the mice via tail vein injection. ...................... 157
LIST OF TABLES

Table 1.1 List of current Clinical Imaging techniques: comparing the advantages and disadvantages ................................................................................................................................. 7

Table 1.2 Summary of the ProCAs described in this thesis ................................................................................................................................. 38

Table 3.1 Final yields of the ProCA1 variants purified using tagless refolding method ........ 78

Table 5.1 Summary of the $K_d$ values of hProCA32 to Tb$^{3+}$, Gd$^{3+}$, Ca$^{2+}$ and Zn$^{2+}$ using various metal binding assay such as metal chelator buffer system (Tb$^{3+}$, Gd$^{3+}$, Ca$^{2+}$) and competition assay using molecular probe FluoZin-1 dye for Zn$^{2+}$ . ............................................................ 160
LIST OF ABBREVIATIONS

ProCA1: Protein based contrast agent, Class I
HER2: human epidermal growth factor receptor 2
Bomb: Bombesin
GRP: gastrin-releasing peptide
GRPR: gastrin-releasing peptide receptor
HER2: human epidermal growth factor receptor 2
hProCA32: Humanized Protein –Based contrast agent, Class 32
E.Coli: Eschericia coli
IPTG: Isopropyl- β-D-thiogalactopyranoside
OD: Optical density
SDS-PAGE: Sodium dodecyl Sulfate Polyacrylamide gel electrophoresis.
EGTA: Ethelyneglycol tetraacetic acid
DTPA: Diethylenetriaminepentaacetic acid
FPLC: Fast protein liquid chromatography
NMR: Nuclear Magnetic Resonance
FRET: Fluorescence Resonance Energy Transfer.
PEG: Polyethylene Glycol.
1. INTRODUCTION

1.1. Cancer

Cancer is one the most common disease world-wide and is caused due to abnormal growth of cells in a particular tissue, which can either stay in the same region or the cells can disburse throughout the body via blood. Unlike normal cells that follow orderly growth, division, and death, cancer cells continue to grow and divide causing the development of uncontrollable masses of abnormal cells[2]. These cells may interfere with the normal bodily function and take up nutrients and resources from healthy cells, leading to death. In such conditions when the cancerous cells invade into healthy cells and tissues through its progression, it is said to be have metastasized, and the condition is referred to as metastasis [3]. The primary goal of most of the researchers and physicians in the field has been the early detection of cancer, which may prevent the disease from being malicious and makes the cancer treatment more effective. Imaging techniques such as X-ray, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) scans, and ultrasound scans are often used to detect a tumor location and the damage that it may have caused in the affected organ.

1.2. Clinical Imaging Modalities

Medical imaging is widely applied in clinical practice referring to the in vivo visualization of images of internal organs of human body. These imaging modalities are used to obtain biological functioning and to measure quantitatively the anatomical and physiological information for clinical and medicinal applications[4]. In particular, imaging techniques have been often used for the diagnosis and prognosis of tumor growth and it metastasis in a human body. Furthermore, practical application of these medical-imaging can allow early detection of
cancerous cells with improved treatment selection. There are plethora of imaging modalities that being used for medical images including X-rays, MRI, CT, PET, Ultrasound, near infrared (NIR), fluorescent imaging of green fluorescence proteins (GFP), Optical Imaging, and Magnetic resonance spectroscopy (MRS). Each of these techniques has their advantages and drawbacks for obtaining physiological and functional information as described in Table 1.1[4]. Various imaging modalities such as X-ray, Positron Emission Tomography (PET), Computed Tomography (CT), Near Infra-Red (NIR), MRI or ultrasound have been utilized for further advancements in the field. These techniques are well studied and explored by the researchers and clinicians. Each has its advantages and disadvantages based on their applications. Optical imaging techniques such as NIR and fluorescence have high sensitivity but lower solution with the lack of deep penetration whereas spectral imaging such as MRI, or PET/SPECT has much deeper penetration. These fluorescent techniques are often used intraoperative medical applications.

Some of the techniques, like X-ray and CT, are used for scanning solid organs, such as bones. However, they use gamma-emitting radioisotopes, which pose dose-dependent risks that prevent further applications for prognosis especially for children. These imaging techniques are not sensitive to soft tissues where cancerous cells reside. PET is another commonly used method for the diagnosis; renders three-dimensional images using radio nuclei tracers generating detectable gamma emissions. PET/SPECT both has high sensitivity and depth penetration; however, they use harmful irradiation and have limited resolution. Ultrasound (does not use radiation) is also widely used for in vivo application, but has limited depth penetration and resolution. Unlike PET and CT, MRI is a noninvasive imaging modality that does not use harmful ionizing radiation to generate two-dimensional and three-dimensional
images of a thin slice through particular regions of the body. In contrast to X-ray, CT, and ultrasound, MRI is not limited to number of scans of an entire body. Along with these properties, MRI also allows for an excellent resolution between normal tissues and cancerous tissues with soft tissue enhancement. Although MRI has no limitation for the depth of tissue penetration, it has low sensitivity and requires the use of a contrast agent that interacts with water proton to increase relaxivity and obtain better images with increased signal-to-noise ratio. Hence, our goal in the development of MRI contrast agents can further enable the medical imaging for early diagnosis and prognosis of cancer. Furthermore, engineering MRI contrast agents with targeted against cancer biomarkers, we can achieve desired sensitivity and specificity and open a new horizon in medical imaging[5, 6].

1.3. Cancer Biomarkers and Targeted Molecular Imaging

Cancer cells tend to express a high level of specific cellular receptors as compared to the normal cells. Some cancerous cells are known to have specific receptors that are over-expressed, and these receptors are used as biomarkers to detect the growth of tumor (Figure 1.1). Much research has been done on cancer biomarkers. Analyzing the cancer biomarkers helps the researcher to understand the diagnosis, prognosis and invasiveness of cancer. There are some cancer receptors that are well known and specific to cancer types(Figure1.2)[7]. For example, the HER family members, epidermal growth factor receptors (EGFR) and HER2 are transmembrane proteins playing a crucial role in a network of multi-layered signal, transduction pathway that results in activation of cellular response such as differentiation, growth and survival, and cell motility[8]. These receptors have relatively low level of expression on normal cells; however, they are overly expressed in variety of human tumors including non-small-cell lung cancer, pancreatic cancers, prostate cancer, and brain cancer [9, 10]. HER2 receptors are
known to overly express on 20-30% of breast cancer and can aggressively metastasize to other organs [8, 11]. EGFR and HER2 receptors are significant prognostic biomarkers, so their signaling pathways during the progression of the disease are studied to understand the mechanistic basis of cancer [10].

Gastrin-releasing protein receptor (GRPR) belongs to the G protein-coupled receptor family that has a typical seven-transmembrane domain, and which is frequently found in pancreas; it is mostly absent in other organs of the body [12]. GRPR is found to have abnormally high expression levels in prostate cancer, gastric cancer, non-small-cell lung cancer, and breast cancer cells compared to normal cells [13]. Furthermore, GRPR appears to have relatively high, (almost 100%) expression on prostate cancer as compared to healthy prostate cells. Therefore, GRPR is widely studied as a biomarker to target cancerous cells. Tracking down the mechanism of cancer progression using biomarkers is a new emerging field that provides answers with improved specificity and sensitivity form any questions in disease diagnosis and targeted treatment. One of the goals of this thesis is to develop a GRPR and bombesin-targeted contrast agent for early diagnosis and prognosis of prostate cancer cells (a detailed explanation can be found in Chapter 3).

Molecular imaging has become a prominent aspect in the direction of cancer diagnosis and effective treatment. Molecular imaging is a technique that probes biomarkers including specific cell surface receptors or surroundings in order to generate in vivo images. This imaging technology combines the biological process with advanced physical and radiological techniques to capture images of various disease pathways at minute cellular levels [14]. The substantial amount of efforts has been directed at developing a non-invasive, high-resolution, in vivo imaging technology by many researchers. Such profound technology can be successfully used
to detect and monitor diseases at an early stage and help us understand its fundamental process. Recent advancements in molecular imaging monitors drug treatment to target disease progression by imaging through ligand receptors on cellular surfaces or using monoclonal antibodies to target receptors [15, 16]. This study can be further performed using the in vivo mechanism of peptide receptor for targeting cancer in hope to better understand the differentiation in diagnosis and monitoring of therapies using biomarker targeted molecular imaging[17]. In this thesis study, we will further develop MRI contrast agents with cancer biomarker targeting capabilities to increase specificity and sensitivity moving toward the clinical applications for early diagnosis.

Figure 1.1 Illustration of various biomarkers expressed under cancerous environment.

Each of these biomarkers plays a critical role in functioning and development of cells. However, these receptors tend to express at higher level under cancerous environment and are specific to the type of cancer, hence are referred to as biomarkers. These biomarkers are often studied for the mechanism, diagnosis and prognosis of cancer.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Disease</th>
<th>Cut Off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>malignant pleural effusion</td>
<td>NA¹</td>
<td>57.5%</td>
<td>78.8%</td>
<td>(Li et al. 2003)</td>
</tr>
<tr>
<td>CEA</td>
<td>peritoneal cancer dissemination</td>
<td>0.5 ng/ml</td>
<td>75.8%</td>
<td>90.8%</td>
<td>(Yamamoto et al. 2004)</td>
</tr>
<tr>
<td>Her-2/neu</td>
<td>stage IV breast cancer</td>
<td>15 ng/mL</td>
<td>40%</td>
<td>98%²</td>
<td>(Cook et al. 2001)</td>
</tr>
<tr>
<td>Bladder Tumor Antigen</td>
<td>urothelial cell carcinoma</td>
<td>NA</td>
<td>52.8%</td>
<td>70%</td>
<td>(Mian et al. 2000)</td>
</tr>
<tr>
<td>Thyro-globulin</td>
<td>thyroid cancer metastasis</td>
<td>2.3 ng/ml³</td>
<td>74.5%</td>
<td>95%</td>
<td>(Lima et al. 2002)</td>
</tr>
<tr>
<td>Alpha-fetoprotein</td>
<td>hepatocellular carcinoma</td>
<td>20 ng/ml</td>
<td>60%</td>
<td>70%</td>
<td>(De Masi et al. 2005)</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate cancer</td>
<td>4.0 ng/mL</td>
<td>46%</td>
<td>91%</td>
<td>(Gann et al. 1995)</td>
</tr>
<tr>
<td>CA 125</td>
<td>non-small cell lung cancer</td>
<td>95 IU/mL</td>
<td>84%</td>
<td>80%</td>
<td>(Dabrowska et al. 2004)</td>
</tr>
<tr>
<td>CA19.9</td>
<td>pancreatic cancer</td>
<td>NA</td>
<td>75%</td>
<td>80%</td>
<td>(Yamaguchi et al. 2004)</td>
</tr>
<tr>
<td>CA 15.3</td>
<td>breast cancer</td>
<td>40 U/ml</td>
<td>58.2%</td>
<td>96.0%</td>
<td>(Ciambellotti et al. 1993)</td>
</tr>
<tr>
<td>Leptin, prolactin, osteopontin, and IGF-II</td>
<td>ovarian cancer</td>
<td>NA</td>
<td>95%</td>
<td>95%</td>
<td>(Mor et al. 2005)</td>
</tr>
<tr>
<td>CD98, fascin, sPigR⁴, and 14-3-3 eta</td>
<td>lung cancer</td>
<td>NA</td>
<td>96%</td>
<td>77%</td>
<td>(Xiao et al. 2005)</td>
</tr>
<tr>
<td>Troponin I</td>
<td>myocardial infarction</td>
<td>0.1 microg/L</td>
<td>93%</td>
<td>81%</td>
<td>(Eagles et al. 2004)</td>
</tr>
<tr>
<td>B-type natriuretic peptide</td>
<td>Congestive heart failure</td>
<td>8 pg/mL</td>
<td>98%</td>
<td>92%</td>
<td>(Dao et al. 2001)</td>
</tr>
</tbody>
</table>

Figure 1.2 Biomarkers that are recognized by FDA considering its sensitivity and specificity in molecular imaging. Adapted from the reference with permission [7]
Table 1.1 List of current Clinical imaging techniques: comparing the advantages and disadvantages

<table>
<thead>
<tr>
<th>Modality</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Common Contrast agents</th>
<th>Example Clinical Applications</th>
</tr>
</thead>
</table>
| CT       | • Unlimited depth penetration  
          • High spatial resolution  
          • Whole-body imaging possible  
          • Short acquisition time (minutes)  
          • Moderately expensive.  
          • Anatomical imaging | • Irradiation exposure  
          • Poor soft tissue contrast  
          • Probably not used for molecular imaging currently only anatomical and functional imaging | • Barium  
          • Iodine  
          • Krypton  
          • Xenon | Tumor Perfusion |
| PET      | • Unlimited depth penetration  
          • Whole-body imaging possible  
          • Quantitative molecular imaging  
          • Can be combined with CT or MRI for anatomical information | • Irradiation exposure  
          • Expensive  
          • Low spatial resolution (1–2 mm, 4–8 mm3)  
          • Long acquisition times | • °C  
          • °F  
          • °Cu  
          • °Ga | °T-FDG-PET for cancer staging  
          • Diagnosis of various diseases |
| MRI      | • Unlimited depth penetration  
          • Whole-body imaging possible  
          • No ionizing irradiation  
          • Excellent soft tissue contrast  
          • High spatial resolution  
          • Established infrastructure | • Expensive  
          • Long acquisition time  
          • Limited sensitivity for detection of molecular contrast agents | Gadolinium (Gd³⁺)  
          • Iron oxide particles  
          • Manganese oxide | Detection of lymph node metastases of prostate cancer  
          • Characterization of focal hepatic lesions |
| Ultrasound | • No ionizing radiation  
              • Real-time imaging/short acquisition time (min)  
              • High spatial resolution  
              • Can be applied externally or internally (endoscopy)  
              • Highly sensitive | • Whole-body imaging not possible  
              • Contrast agents currently limited to vascularity  
              • Operator dependency | Contrast Microbubbles | Characterization of focal liver lesions  
              • Echocardiography  
              • Tumor perfusion of cancer |
| Optical  | • No ionizing radiation  
          • Real-time imaging/short acquisition time (sec-min)  
          • High spatial resolution  
          • Can be applied externally or internally (endoscopy)  
          • Highly quantitative & Sensitive | Limited depth penetration (≤ 1 cm)  
          • Whole-body imaging not possible | • Whole-body imaging not possible | OCT imaging of atherosclerosis  
          • OCT imaging for colonoscopy screening  
          • Raman imaging of skin cancer |

*Adapted and modified with permission from the reference [4]
1.4. Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging, developed from the basic principles of nuclear magnetic resonance, is an important medical imaging technique that can give biological, chemical and functional information about a body. MRI is one of the most powerful imaging techniques that provide non-invasive diagnostic methods in current clinical and research field. MRI is a promising technique due to its significant ability to generate three-dimensional images of the soft tissues with in-depth tissue penetration. MRI measures the magnetic properties of water hydrogen within an external magnetic field (B\textsubscript{0}), as they interact with radio frequency (R\textsubscript{f}) with electronic, magnetic waves. MRI thus detects signals generated by hydrogen atoms, which are abundant in the form of water molecules comprising 70% of human body, as the signal remain uninfluenced by other predominant biological compounds such as \textsuperscript{12}C, \textsuperscript{16}O, and \textsuperscript{14}N\cite{18, 19}.

As shown in Figure 1.4, MRI images are generated by relaxation rates of hydrogen nuclei containing single proton, which undergoes multiple spin states such as magnetic and angular moment, generating magnetization properties. Nuclei containing even number of protons and neutrons have zero net magnetization (\textsuperscript{12}C or \textsuperscript{14}N) due to cancellation of paired spin, while hydrogen nuclei containing an odd number of protons, have an angular momentum with net spin of ±1/2 (magnetic quantum number) that are unpaired, giving rise to magnetic properties due to rotational and vibrational motion. Hydrogen nuclei are in a low energy state and have random orientation in the absence of external magnetic field (B\textsubscript{0}). However, during the MRI scans, these hydrogen nuclei present in various organs of the body aligns in the presence of the magnetic field generating an overall net magnetization. In general, when a uniform external magnetic field (B\textsubscript{0}) is applied, the hydrogen nuclei align either parallel or anti-parallel to the magnetic field creating a net magnetic moment (M) (Figure 1.3). Under the external
magnetic field, these nuclei rotate or precess along the applied magnetic field and the frequency at which the nuclei rotate or precesses known as Larmor Frequency ($\omega$). Larmor frequency depends on the gyromagnetic ratio ($\gamma$), ratio of angular momentum (J) and magnetic field ($B_0$) (Equation 1-2).

The following equation describes the angular momentum (J) and the magnetic moment where $m$ is the mass, $v$ is the velocity and $r$ is the radius of the proton.

$$J = m\omega = mvr \quad \text{Equation 1-1}$$

$$\omega = \gamma B_0 \quad \text{Equation 1-2}$$

Subsequently, when a pulse of radiofrequency (RF) electromagnetic radiation is applied in the direction perpendicular to the magnetic field, the hydrogen nuclei absorbs this energy causing it to perturb or to tilt from the magnetic field (Figure 1.3). Once the radiofrequency pulse is removed, the nuclei in its high energy state decay back to the lower energy state, and this process is called relaxation. The MRI measures the signal produced by the nuclei as it relaxes or reorients back to its initial position relative to the magnetic field; from the aggregates of these signals it generates an image. The time it takes for the nuclei to return to its original orientation along the magnetic field corresponds to the enhancements in the MRI images. There are two relaxation processes that occur simultaneously when the proton relaxes back to its original orientation: longitudinal (spin-lattice) relaxation ($r_1$) and transverse (spin-spin) relaxation ($r_2$)[20, 21]. The time it takes for the nuclei to realign back to its initial orientation in the magnetic field along ZY plane is called spin-lattice or longitudinal time $T_1$. As illustrated in Figure 1.4A, during application of an RF pulse, the proton precesses with an angle of $\theta$. Once the RF pulse is removed, the proton resumes back to its low energy state (Z-axis) and the angle
\(\theta\) decreases. This process is referred to as T₁ relaxation time. Simultaneously, the nuclei spin dephasing along XY plane around the Z-axis. This dephasing process is called spin-spin relaxation time T₂ (Figure 1.4B).

One of major challenges of clinical MRI for human patients is its low sensitivity and high background. Since most of human body is made of water, these relaxation rates of protons associated with the tissue observed have minute differences with an enormous background water signal. This leads to lower sensitivity in differentiating between the tissues of the interior of two organs as compared to soft tissue. In order to make MRI suitable with increased sensitivity for organ related diseases, contrast agents are widely used. However, there is a pressing need to develop sensitivity MRI contrast agents to extend the clinical applications of MRI. The objective of this thesis is to develop MR contrast agent with high relaxivity to enable molecular imaging of biomarkers. In addition, we also improve their translational potentials by reducing immunogenicity.
Figure 1.3 Schematic representation of nuclei under magnetic field and their relaxation process.

The nuclei in its original state have random orientation, when a magnetic field is applied the spin states of these nuclei align parallel or anti parallel. When radiofrequency pulse is applied, in the direction perpendicular to the magnetic field, the hydrogen nuclei absorbs this energy causing it to perturb or tilting from the magnetic field. Once the radiofrequency pulse is removed, the nuclei in its high energy state decay back to the lower energy state along the Z-axis, and this process is called relaxation. There are two relaxation processes that occur simultaneously when the proton relaxes back to its original orientation: longitudinal (spin-lattice) relaxation ($r_1$) and transverse (spin-spin) relaxation ($r_2$).
Figure 1.4 Diagram depicting the process of (A) R1 relaxivity and (B) R2 relaxivity.

There are two relaxation processes that occur simultaneously when the proton relaxes back to its original orientation: longitudinal (spin-lattice) relaxation ($r_1$) and transverse (spin-spin) relaxation ($r_2$). The time it takes for the nuclei to realign back to its initial orientation in the magnetic field along ZY plane is called spin lattice or longitudinal time $T_1$. During application of an RF pulse, the proton precesses with an angle of $\theta$. Once the RF pulse is removed, the proton resumes back to its low energy state (Z-axis) and the angle $\theta$ decreases. This process is referred to as $T_1$ relaxation time. Simultaneously, the nuclei spin de-phasing along XY plane around the Z-axis. This de-phasing process is called spin-spin relaxation time $T_2$. 
1.5. Contrast Agents in MRI

In order to attain MRI scans with sufficient sensitivity and enhancement, contrast agents are often used. Around 30-50% of clinical MRI scans are obtained by injecting MRI contrast agents intravenously [22-24]. Contrast agents enhance the signal corresponding to the relaxation rate of water proton. Contrast agents can enhance the imaging signals along with distinguishing the diseased organ from the regular ones. A typical MRI contrast agent shortens $T_1$ and $T_2$ relaxation time of surrounding water molecules and thereby enhances the contrast of the images. This is described as relaxivities, $r_1$ and $r_2$.

Depending on their physical properties, MRI contrast agents have various categories. “T1 weighted” contrast agents are capable of reducing both longitudinal ($T_1$) and transverse ($T_2$) relaxation time of water hydrogen while “T2 weighted” contrast agents have shorter transverse ($T_2$) relaxation time as compared to longitudinal ($T_1$) (Figure 1.3 and 1.4). T1 weighted contrast agents brighten the image by producing positive contrast (gadolinium complexes) whereas T2 weighted contrast agents darken the images by creating negative contrast as with iron oxide contrast agents. The sensitivity and effectiveness of a contrast agent rely on how well it can shorten the proton relaxation. These properties are achieved by using paramagnetic, ferromagnetic or super paramagnetic metal ions containing unpaired electrons as contrasting reagents. Gadolinium ($\text{Gd}^{3+}$), a trivalent lanthanide metal ion with seven unpaired electrons, high magnetic moments, long electronic relaxation times due to its symmetric s-state, is the most frequently used metal ion. Gadolinium-based contrast agents are T1 weighted because of its capability to shorten the longitudinal ($T_1$) relaxation time. The interactions of seven unpaired electrons of gadolinium with water protons generate a brighter contrast. The direct coordination of water to the gadolinium center contributes to the inner sphere relaxation.
whereas the surrounding bulk solvent water contributes to the outer sphere relaxation. There are many parameters that affect the relaxivity such as rotational correlation time, distance between proton and center of gadolinium, water exchange rate with gadolinium (kexor/τm), the electronic properties of the gadolinium ion (T1e) and hydration number of the inner and outer sphere (q). Other parameters that may affect the relaxation process are the strength of the magnetic field, and viscosity of the solution. Manipulating these parameters can help in the development of better contrast agents as described in the following Equation 1-3 where 1/Tc1 is the inverse Larmor frequency.

\[
\frac{1}{T_{c1}} = \frac{1}{\tau_R} + \frac{1}{T_{1e}} + \frac{1}{\tau_m}
\]

Equation 1-3

\[
R_{1,2} = \frac{\left( \frac{1}{T_{1,2} \text{Sample}} - \frac{1}{T_{1,2} \text{Buffer}} \right)}{[\text{Gd}^{3+} ]}
\]

Equation 1-4

### 1.5.1. Current MRI Contrast Agents and their limitations

Free gadolinium is highly toxic by itself, for instance, the toxicity in mice is with an LD\textsubscript{50} of 0.2 mmol/kg. Thus free Gd\textsuperscript{3+} must be coupled with a chelator that functions as a carrier to encapsulate it \textit{in vivo}. Current clinically approved contrast agents have one Gd\textsuperscript{3+} that is incorporated into a small organic chelator such as diethylene triamine penta acetic acid (DTPA), tetraazacyclododecane tetraacetic acid (DOTA) etc. (Figure 1.5)[25]. The primary structures of these chelators vary from linear to cyclic structures. For instance, the thermostability and dynamics of macro-cyclic ligand Gd-DOTA is relatively higher than that linear of Gd-DTPA[22, 26]. These FDA-approved contrast agents have relaxivities of 4 – 5 mM\textsuperscript{-1} s\textsuperscript{-1}, however; theoretically they are expected to have relaxivities of around 100 mM\textsuperscript{-1} s\textsuperscript{-1}. Due to slow water exchange and fast molecular rotation time (100 ps) of small chelators, the
possibility of achieving such high theoretical relaxivity 100 mM$^{-1}$s$^{-1}$ is significantly compromised[22]. Along with this, they have short half-lives, so very high concentration (> 0.1 mM range), attained with repeated injection (at 0.1-0.3 mM/kg), is required in order to detect changes in contrast caused by proton relaxation[6, 24, 27]. Such high concentrations of free gadolinium, lower blood retention and decreased \textit{in vivo} relaxivities may increase the risk of causing nephrogenic systemic fibrosis (NSF), which affects the kidney. Studies have shown that due to weak binding between Gd$^{3+}$ and contrast agents leads to accumulation of free gadolinium and free contrast agents in the kidney causes lack of proper excretion, and consequentially causes renal failure.[28] There are certain restrictions imposed by the FDA in the dosage and applications of these contrast agents, depending on the severity of the harm they may cause shown in \textbf{Figure 1.6}. Moreover, these contrast agents have poor organ specificity and lack the targeting capability. Thus, there is an urgent need to develop a contrast agent with high relaxivity, higher rotational correlation time, better retention and potential targeting capability.
Figure 1.5 Commercially available, clinically used and FDA approved MRI contrast agents. Adapted with permission from reference [25]
<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Trade name</th>
<th>Manufacturer</th>
<th>Charge and Chemical structure</th>
<th>Conditional stability at pH 7.4 (log)</th>
<th>Excess chelate (mg/ml)</th>
<th>Thermodynamic stability constant (log)</th>
<th>Kinetic stability*</th>
<th>Body region(s) approved</th>
<th>Approval status</th>
<th>Approved doses for imaging (mmol/kg)</th>
<th>Approved doses for children (mmol/kg)</th>
<th>NSF related to gadolinium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadodiamide</td>
<td>Omniscan</td>
<td>GE Healthcare</td>
<td>Non ionic linear</td>
<td>14.9</td>
<td>12</td>
<td>16.9</td>
<td>35 s</td>
<td>CNS, whole body</td>
<td>USA, EU, Japan</td>
<td>Body 0.1-0.3, CNS 0.1-0.3, MRA 0.1-0.3</td>
<td>From 6 months: 0.1</td>
<td>Yes (180 cases worldwide)</td>
</tr>
<tr>
<td>Gadopentetate dimeglumine</td>
<td>Magnevist</td>
<td>Bayer Schering Pharma</td>
<td>Ionic linear</td>
<td>17.7</td>
<td>0.4</td>
<td>22.1</td>
<td>10 min</td>
<td>CNS, whole body</td>
<td>USA, EU, Japan</td>
<td>Body 0.1, CNS 0.1-0.2, MRA 0.1-0.3</td>
<td>0.1 (doses of 0.2 may be used if necessary for children older than 2 years)</td>
<td>Yes (78 cases worldwide)</td>
</tr>
<tr>
<td>Gadobenate dimeglumine</td>
<td>Multihance</td>
<td>Bracco</td>
<td>Ionic linear</td>
<td>16.9</td>
<td>None</td>
<td>22.6</td>
<td>N/A</td>
<td>CNS, liver</td>
<td>USA, EU</td>
<td>Liver 0.05, CNS 0.1, MRA not approved</td>
<td>Not approved &lt;18 years</td>
<td>Yes (1 case in a patient coadministered Omniscan)</td>
</tr>
<tr>
<td>Gadoveratamid</td>
<td>OptiMARK</td>
<td>Tyco</td>
<td>Non ionic linear</td>
<td>15.0</td>
<td>28.4</td>
<td>16.6</td>
<td>N/A</td>
<td>CNS, liver</td>
<td>USA</td>
<td>Body 0.1, CNS 0.1, MRA not approved</td>
<td>Not approved &lt;18 years</td>
<td>Yes</td>
</tr>
<tr>
<td>Gadoterate meglumine</td>
<td>Dotarem</td>
<td>Guerbet</td>
<td>Ionic cyclic</td>
<td>18.8</td>
<td>None</td>
<td>25.8</td>
<td>&gt;1 month</td>
<td>CNS, whole body</td>
<td>EU</td>
<td>Body 0.1, CNS 0.1-0.3, MRA 0.2</td>
<td>0.1</td>
<td>No</td>
</tr>
<tr>
<td>Gadoteridol</td>
<td>ProHance</td>
<td>Bracco</td>
<td>Non ionic cyclic</td>
<td>17.1</td>
<td>0.23</td>
<td>23.8</td>
<td>3 h</td>
<td>CNS, whole body</td>
<td>USA, EU, Japan</td>
<td>Body 0.1-0.3, CNS 0.1-0.3, MRA not approved</td>
<td>0.1 from 2 years, caution 6 months to 2 years, contraindicated &lt;6months</td>
<td>No</td>
</tr>
<tr>
<td>Gadobutrol</td>
<td>Gadovist</td>
<td>Bayer Schering Pharma</td>
<td>Non – ionic cyclic</td>
<td>N/A</td>
<td>N/A</td>
<td>21.8</td>
<td>5 min</td>
<td>CNS, MRA</td>
<td>EU, Canada</td>
<td>Body not approved, CNS 0.1-0.3, MRA (imaging 1 FOV) 0.1-0.15, MRA (imaging &gt;1 FOV) 0.2-0.3</td>
<td>Not approved &lt;18 years</td>
<td>No</td>
</tr>
<tr>
<td>Gadoxetic acid</td>
<td>Primovist</td>
<td>Bayer Schering Pharma</td>
<td>Ionic linear</td>
<td>N/A</td>
<td>N/A</td>
<td>23.5</td>
<td>N/A</td>
<td>Liver</td>
<td>USA, EU</td>
<td>Body 25 µmol/kg or 0.1 ml/kg, CNS not approved, MRA not approved</td>
<td>Not approved &lt;18 years</td>
<td>No</td>
</tr>
<tr>
<td>Gadofosveset</td>
<td>Vasovist</td>
<td>Bayer Schering Pharma</td>
<td>Ionic linear</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Abdominal and limb vessels</td>
<td>EU</td>
<td>MRA 0.03</td>
<td>Not approved &lt;18 years</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 1.6 List of contrast agents with certain restrictions and dosage approval worldwide. Adapted with permission from reference [28]
1.5.2. Criteria for designing Contrast Agents

As discussed above, in order to develop a clinically reliable protein-based MRI contrast agent, it must fulfill certain criterion including high stability and solubility, appropriate molecular weight, tolerance to mutations, high relaxivity, strong Gd$^{3+}$ stability and metal selectivity, no toxicity, and, last but not least, the production must be cost effective and time efficient. In order to evaluate high quality contrast agent with less toxicity for clinical applications, it must possess high in vivo relaxivity, that it must shorten the relaxation times (T1 and T2) to achieve maximum enhancement with a low dosage. A contrast agent must have strong affinity and selectivity for the lanthanide metals (gadolinium) over other physiological metals such as calcium, zinc and magnesium that can possibly compete with gadolinium and release it out of the contrast agent causing toxicity and may hinder the outcome. It must also have excellent in vivo stability without any interactions with proteolytic enzymes, proteins and other physiological components that can cause degradation or cleavage, which can in return increase blood retention time and minimize the dosage requirement. It must have the appropriate size for fast circulation, renal filtration without any accumulation in the kidney. Most importantly, it must have reduced immunological effects. Finally, molecular targeting of diseased cells is highly desired to enhance the specificity of contrast agents to attain high spatial resolution using MRI. Our lab has taken these factors into account and designed a suitable protein-based contrast agent, and further optimized its properties by targeting it to cancer biomarkers.
1.6. Designing Protein-based MRI contrast agents – a novel approach

Yang Lab has developed a novel class of MRI contrast agents using proteins. A Gd$^{3+}$ binding site was created on to a scaffold protein frame to increase relaxivity by controlling correlation time, water in the coordination shell and addition contribution of the secondary shell to relaxivity. The protein-based contrast agents, ProCAs were achieved based on our previous studies on designing and engineering metal-binding sites in proteins and protein folding. Various computational and protein engineering strategies have been developed to introduce metal binding pockets and alter the coordination without disrupting the protein folding and compact tertiary structures. In addition, well-folded scaffold with appropriate size of about 2-3 nm (approximate molecular weight 10-12 kDa) have good tissue penetration, adequate circulation time and are excreted through the kidney. Further, the recent development in methodologies for determining structural and dynamic properties with sophisticated techniques such as high resolution NMR have made it possible for designing MRI contrast agents with desired metal (Gd$^{3+}$) binding pocket and relaxation properties. Fifth, protein can be modified and conjugated with moieties that can target diseased biomarkers.

Protein based contrast agent, ProCAs have several advantages over the commercial contrast agents. Proteins engineering can generate a stronger coordination in the pre-existing binding pocket to improve its metal-binding affinities and selectivity and can be used as scaffold protein. When gadolinium binds to these altered scaffold proteins, they can probe in increasing the amount of bulk water in the surroundings. The Gd$^{3+}$ interacts with the surrounding bulk water with one inner sphere water, three-second sphere water molecules and several outer sphere water molecules (Figure 1.7A)[22]. With an optimal increase in molecular size by conjugation of ProCA and with hydrophilic PEG reagents, the water exchange rate can
be improved dramatically, which in turn increases the relaxivity. Conjugation with PEG increases the tumbling or rotational correlation time $\tau_r$, which is dependent on the molecular size, and plays a major role in the enhancement of relaxation properties (Figure 1.7A). As illustrated in Figure 1.7B, the simulations of magnetic field dependent relaxivities show that the protein contrast agent has a rotational correlation time of 10 ns, which can increase the relaxivities up to 10-20 folds by optimizing these parameters such as tumbling rate or rotational correlation time, water exchange between inner and outer sphere of gadolinium. Newly developed ProCAs that are well packed and have the optimal molecular size of about 10-12 kDa and can be quickly excreted from the kidney. In addition, ProCAs can also be modified and engineered with targeting moieties for various diseased biomarkers. Subsequently, our lab has developed two generations of protein-based MRI contrast agents, with various targeting capabilities that specifically target highly expressed biomarkers on cancerous cells.
Figure 1.7 Schematic representation of the factors that can be optimized to improve the properties of protein based contrast agent.

(A) When gadolinium binds to these altered scaffold proteins, they can probe in increasing the amount of bulk water in the surroundings. Gd$^{3+}$ interacts with the surrounding bulk water with one inner sphere water, three-second sphere water molecules and several outer sphere water molecules. Conjugation with hydrophilic PEG reagent with optimum molecular weight increases the tumbling or rotational correlation time $\tau_R$, which is dependent on the molecular size, and plays a significant role in enhancement of relaxation properties. (B) The simulations of magnetic field dependent relaxivities show that the protein contrast agent has a rotational correlation time of 10 ns, which can increase the relaxivities up to 10-20 folds by optimizing these parameters such as tumbling rate or rotational correlation time, water exchange between inner and outer sphere of gadolinium. Adapted with permission from reference[22]
1.6.1. ProCA1

Previously, Dr. Jenny Yang’s lab has successfully overcome the above-mentioned challenges and developed a series of novel classes of ProCAs. The first important step is the selection of a scaffold protein that is tolerant to mutations and maintains its stability and rigid structure when subjected to physiological environment [29]. In order to develop protein-based contrast agents with high metal binding affinity, various proteins and small chelators were studied for metal binding sites. Similar to Ca$^{2+}$ and lanthanides such as Tb$^{3+}$, and Eu$^{3+}$, Gd$^{3+}$ also uses oxygen and nitrogen as ligand; however oxygen has higher preference as a ligand in a protein molecule. Surveys for metal binding coordination of x-ray structures of calcium binding proteins with calcium and lanthanide ions suggested that the average coordination number of these lanthanide ions in an intrinsic protein EF-hand site was 7.2[30]. It is well known that these lanthanides, Tb$^{3+}$ and Eu$^{3+}$ possess similar ionic radii and coordination number as Ca$^{2+}$ and are often used as fluorescent probes to determine Ca$^{2+}$ binding affinities. In fact, many biological functioning such as conversion of prothrombin to thrombin and activation of hemocyaninethat are associated with calcium binding proteins can be efficiently performed by substituting Tb$^{3+}$ in the system[31, 32]. Hence, Calcium binding proteins can be an excellent candidate for the selection of scaffold proteins. Proteins containing EF-hand motif as calcium-binding pockets can form penta bipyramid structure with oxygen ligand and thus can be used as Gd$^{3+}$ binding pocket. EF-hand motif can be engineered in any desired scaffold protein to construct as a Gd$^{3+}$ binding pocket. Previously, Dr. Yang’s lab introduced a de novo designed calcium-binding pocket onto a non-calcium binding protein, domain 1 of rat CD2, which demonstrated selective calcium binding over magnesium with dissociation constant of 50 μM that is very much similar to naturally existing extracellular calcium-binding proteins[33, 34]. Domain 1 of rat CD2 is a
derivation of cell adhesion 2 (CD2), originating from T-cell receptor in the immunoglobulin response system. It maintains its original conformation a β-sheet topology, under varying pH and temperature. [35] Initially, a calcium-binding pocket was constructed with five mutations to test its binding capabilities and thermostability. This construct was further optimized to improve its capabilities for strong binding with lanthanides such as Tb$^{3+}$ which showed a dissociation constant less than 1 μM. [36, 37]. Modifying this calcium-binding site further, a novel design approach has been introduced to engineer a gadolinium-binding pocket onto a stable protein, domain 1 of rat CD2. For constructing the Gd$^{3+}$ binding pocket, oxygen from the side chains of Glu15, Glu 56, Asp 58, Asp 62, and Asp 64 was used as a ligand that comes together from the different regions of the protein sequence and forms a binding pocket (Figure 1.8 A). In order to allow fast water exchange rate between the metal ion and the solvent, one coordination position is kept open. (Figure 1.8 A) After rigorous simulations and efforts, Domain 1 of rat CD2 was optimized to have high relaxivity, r1 = 117 mM$^{-1}$ s$^{-1}$ compared to Gd-DTPA r1 = 5.4 mM$^{-1}$ s$^{-1}$ [22, 29] as ProCA1. The molecular size of ProCA1 is about 11 kDa, suitable for optimal rotational rate and longer blood retention with easy renal excretion. As mentioned earlier, ProCA1 is conjugated with 12 kDa PEG to further improve its in vivo properties as contrast agent and enhancement has been achieved (Figure 1.8 B)[22, 38]. Previous lab members have successfully targeted ProCA1 by introducing various moieties that can target HER-2 receptor on breast cancer cells[6] and GRPR receptors on prostate cancer biomarkers.(unpublished data)

Senior lab member, Dr. Qiao has successfully developed targeted ProCA1 by incorporating into an affibody that can target to these overexpressed HER2 receptors on breast cancer cells. Affibody (7 kDa) is obtained from the Z- domain of Protein A, and acts as an antibody that targets these receptors on cell surface. Affibody was grafted onto the C-terminal
of ProCA1 with a flexible linker, GGSGG that prevents steric hindrance between two moieties. Two variants were constructed to target EGFR and HER2 receptors; both demonstrated high relaxivities, sensitivity and excellent targeting capabilities as compared to the commercial contrast agents[6]

As shown in Figure 1.9, GRPR are known to have high expression on prostate cancer cells [27]. In order to develop molecular imaging probe to target prostate cancer, ProCA1 has also been linked with various analogs of GRP peptides via similar grafting approach to target against GRP receptors as discusses in section 1.3

N15E, L58D, K64D

Binding pocket: E15, E56, D58, D62, E64
Figure 1.8 Model structure of ProCA1 with binding gadolinium binding pocket and its coordination. (Generated by Chimera UCSF).

(B) Oxygen ligands from the side chain of Glu15, Glu56, Asp 58, Asp62 and Asp64 are used at different stretches of the protein sequence of domain 1 of CD2 (pdb:1hng). One position of metal geometry is left open to allow fast water exchange between solvent and metal. (C) MRI scans of mice organ enhanced (b) 20 minutes, (c) 3 hours, (d) 24 hours after the injection of ProCA1 compared with (a) prescan, (e) the intensities of kidney and liver before and after injection of ProCA1. (B) and (C) Adapted with permission from reference[38]
1.6.2. *Grafting approach on ProCA1 for targeting GRPR*

As discussed in Section 1.3 and Figure 1.9, gastrin-releasing peptide (GRP) is a natural ligand that can target these overly expressed GRPR on the tumor cells in the prostate. GRP is a peptide that is twenty-seven residues long and belongs to the family of bombesin like peptides\[1\]. Bombesin is a fourteen amino acid long peptide, which was originally isolated from a frog’s skin. GRP -27 is similar to bombesin-14, which has conserved C-terminus (Figure 1.9). The conserved region presumably be playing an important role. Both peptides have high binding affinity (and the targeting ability) to GRPR expressed on prostate tumor surface. Various analogs of GRP and bombesin peptides have been studied: bombesin-14 has stronger binding affinity for GRPR followed by GRP$_{1-27}$ (Figure 1.9). Thus, gastrin-releasing peptide and bombesin can be used to study, monitor and target prostate cancer.
Previously, a 10 amino acid peptide chain from the C-terminus of GRP (GRP1-10) has been inserted into ProCA1 sequence by two ways: by conjugating the peptide at C-terminal of ProCA1 (ProCA1.GRPC) and by grafting the GRP peptide between Glycine52 and Glycine53 in the ProCA1 sequence (ProCA1.GRP52) (Figure 1.10) (designed by PhD student Lexia Wei)[27]. Although, both targeted variants demonstrated high relaxivities; ProCA1.GRP (52) has a significantly stronger binding affinity for GRPR receptor on prostate cancer cell lines [27]. These results suggest that grafting a small peptide into ProCA1 does not affect its
properties as a contrast agent and indeed does not disrupt its original confirmations and folding. Now that it is established that grafting approach demonstrates better targeting abilities, remaining analogs of GRP and bombesin could be examined for targeting GRPR. In order to further optimize and improve the \textit{in vivo} molecular targeting of ProCA1.GRP 52, ProCA1 conjugated with ten amino acid peptide of GRP to GRPR on prostate cancer, Dr. Fan Pu has developed two more variants keeping the targeting ability of bombesin14. Further in the thesis ProCA1.GRP52 is referred to as ProCA1.G10 (or GRP\textsubscript{1-10}). Hypothetically, the conserved region of 10 amino acids in bombesin-14 (bomb\textsubscript{1-10}) can have similar targeting capability as GRP\textsubscript{1-10} (G10) peptide. To test this hypothesis, two peptides bombesin\textsubscript{1-10} with 10 conserved residues (B10) and bombesin-14 (B14), the entire peptide chain were grafted between Gly52 and Gly53 via the GGSGG flexible linker referred as ProCA1.B10 and ProCA1.B14 respectively (\textbf{Figure 1.11})

![Figure 1.10 Two approached: engineering of targeting sequence via (left) inserting it at C-terminal of ProCA1 (ProCA1.GRPC) and (Right) grafting into the ProCA1 sequence between Gly52 and Gly53 (ProCA1.GRP (52)). Adapted with permission from reference [27]]
1.6.3. ProCA\textsubscript{32}

As discussed earlier, Dr. Jenny Yang’s lab has developed a series of novel protein-based MRI contrast agents with engineered Gd\textsuperscript{3+} binding sites into a scaffold protein CD2. The first generation of ProCAs, demonstrated improved relaxivity, sensitivity, selectivity, stability and high targeting capability as compared to the commercially available contrast agents. Although ProCA\textsubscript{1} demonstrated excellent $r_1$ and $r_2$ relaxivities it exhibited relatively weaker Gd\textsuperscript{3+} binding constants than the dissociation constant of the commercial contrast agent Gd-DTPA ($1.86 \times 10^{-21}$ M), which is extremely important for \textit{in vivo} application as an MRI contrast agent to further. So to improvise the stability by selecting a thermodynamically stable scaffold proteins and metal binding by additional binding sites to increase the payload, a series of the third generation of ProCAs, ProCA\textsubscript{3} were designed by Dr. Shenghui Xue. A new scaffold protein, rat $\alpha$
parvalbumin (PV) was selected due to many advantages associated with its physical and functional properties. Firstly, parvalbumin is a small rigid and globular protein with a molecular weight of 11-12 kDa Parvalbumin. PV is a part of calcium-binding protein family, consisting of two Ca$^{2+}$ binding sites, and naturally functions as a cytosolic Ca$^{2+}$ buffer [40]. As discussed earlier, EF-hand motif can form a pentagonal bipyramid structure using oxygen as a ligand and can be an excellent tool for gadolinium binding. Furthermore, Henzl et al. has shown that the calcium binding affinity of parvalbumin can be altered by performing mutations of the key residues in these two binding sites and can be as low as 10$^{-9}$ M.[41-43] The presence of two metal binding sites (AB and CD sites) gives an advantage for designing a potential ProCA with more payload. Although lanthanides possess higher charge, due to similarities in the coordination numbers and with ionic radii almost similar to calcium’s lanthanides have a high affinity for Ca$^{2+}$ sites on biological molecules and stronger interactions with surrounding water molecules.[44] Interaction with surrounding water molecules is significantly important for optimizing the relaxation property of a contrast agent. Most importantly parvalbumin has a strong thermostability, high solubility, and resistance to protease cleavage and is tolerant to mutations. Keeping the above parameters in mind, different mutants of ProCA3, with PV as scaffold protein, were generated to engineer a stronger Gd$^{3+}$ binding site.

Wild-type parvalbumin has two calcium binding sites that are EF-hands. EF-hand site 1 (CD) is formed by four carboxyl ligands (D52, D54, E50, and E63) and one non-charged oxygen ligand (S56) that directly interact with Ca$^{2+}$. EF-hand site II (EF) has carboxyl groups (D91, D93, D95, and E102) and uses G99 as water ligand to hold Ca$^{2+}$ in the binding pocket. As mentioned earlier, calcium and lanthanide ion binding affinities can be selectively improved by introducing a negative charge in the binding pocket. Various mutations were performed and
tested for its metal binding and relaxivity. ProCA32, one of the engineered contrast agents that contain two EF hand binding sites located in helices EF and CD and one additional EF-hand motif AB, with one site mutation within the CD binding pocket (S56D). An increased metal selectivity for Ca$^{2+}$ or lanthanides can be achieved by increasing the negative charge in the metal coordination shell[44]. Hence, negatively charged ligand with S56D mutation in the CD motif was performed to increase the Gd$^{3+}$ binding affinity. Furthermore, an additional mutation (F103W), adjacent to the CD binding pocket, was made to enable fluorescence emission analysis. ProCA32 demonstrated stable serum stability, has low toxicity and behaves as a ratiometric T1/T2 weighted contrast agent. The interaction with gadolinium and other metals were tested through a series of biophysical applications described in Dr. Shenghui Xue’s dissertation (Figure 1.12). In order to consider ProCA32 as a suitable protein drug for the diagnostic and therapeutic of the human disease, it must fulfill certain requirements. There are several factors to be considered for the proper activity of the protein drugs in vivo. Based on these studies, the next step would be to develop a humanized ProCA32 with similar mutations. As shown in the Figure 1.13, there are nine residue differences between the wild type rat PV and wild type human PV. Among these nine residues, only one residue, Asp located in the binding pocket, EF-hand site II (highlighted in cyan) was mutated to Glu (D101E) to make similar binding pocket as ProCA32. The remaining two mutations, S56D located in the binding CD binding site and F103W (highlighted in red) were performed to further achieve similar metal binding and relaxation as compared to rat ProCA32 (Figure 1.13).
Figure 1.12 A Cartoon model of rat ProCA32 and MRI images.

(A) Model structure of ProCA32 using Chimera UCSF sequence derived from x-ray crystal structure.[45] (B) The MRI image of mice before and 50 minutes after injection of ProCA32 (unpublished data) [39] the contrast agent clearly enhances the liver and blood vessels as it is being distributed out from liver via blood.
Figure 1.13 Protein sequence alignment of hCA32 with rat ProCA32, wild type rat Parvalbumin (ProCA30), wild type human parvalbumin (hCA30).

(Top right) Model structure of wild type human CA32 using Chimera UCSF sequence derived from x-ray crystal structure, 1RK9\cite{46}. (Top left) Two Gd\(^{3+}\) binding pockets were generated on the CD and EF-loop of helix-loop-helix structure in wild type human PV by mutations at (CD site I) S56D and D101E (EF site II). Orange dot is for Gd\(^{3+}\) metal in the center, purple dots surrounding the metal ion correspond to the charged oxygen ligand for Gd\(^{3+}\) from carboxyl group, black dot indicates the oxygen ligand for Gd\(^{3+}\) from protein main chain and blue dot in site II is for the oxygen ligand from the surrounding water molecule. (Bottom) the Protein sequence alignment of the wild type rat and human PV with rat ProCA32 and hProCA32. The ones highlighted in yellow are the amino acid difference between rat and human PV. The ones highlighted in red indicate the key mutation in rat and human ProCA32 (1) to improve metal binding affinity (S56D) and 2) for studying the fluorescence assay (F103W).
Figure 1.14 Overlay of hCA32 and rat ProCA32 using UCSF Chimera

Structure in yellow is rat ProCA32, 1rwy with mutation S56D and F103W performed using chimera UCSF[45] and purple is hProCA32, 1RK9 with mutation S56d, E101D and F103W using chimera UCSF [46].
1.7. Additional challenges in developing a Protein Based MRI Contrast Agents for clinical applications

In developing a novel class of contrast agent that can be utilized in clinical applications, there are several hurdles that need to be crossed. Primarily, the production of these newly engineered contrast agents must be optimized to get well-folded, stable and high yield of ProCAs for further in vivo studies. Purification protocol that can be easily applied into large scale or industrial setting must be optimized for ProCA1 variants and humanized ProCA32 (hCA32). To further move into the next phase of pre-clinical trials of therapeutic and diagnostic drug discovery, the contrast agent must in be used for larger animal followed by clinical trials. When non-human analogs of drugs are used, they tend to initiate immune response.

1.7.1. Approaches to reduce Immunogenicity

The main aim of developing these new generations of protein based contrast agents is to address the clinical relevance by reducing its immunogenicity. Immunogenicity of the protein therapeutics remains one of the most challenging aspects in the field of drug discovery and therapeutics industries. Many of these non-human protein sequences like fusion proteins, engineered protein variants that are derived via bacterial medium and other monoclonal antibodies can possess higher clinical performances, novel functions and have potency to become pioneers of drug therapeutics, however, due to the major barrier caused by immunogenicity such remarkable preliminary discoveries remains futile[47]. When these exogenous therapeutic protein drugs are introduced into the human body, the immune system tends to form antibodies against it. Thus, the most essential requirement to address the immunogenicity is to reduce the immunogenicity along with maintaining the desired structural and functional significance. There are various means to address immunogenicity including
modification by PEGylation, site-specific mutagenesis, exon shuffling or humanization[48]. Recently, a new method has been developed to target immunogenicity here the therapeutic drug is modified with sugar, glycosylation [49].

Humanization of desired non-human protein will essentially increase its survival into immune system and reduce the immunogenicity response. Along with reduced immunogenicity, to allow protein therapeutics for successful application it is necessary to optimize and consider several factors such as solubility, stability, pharmacokinetics, and pharmacodynamics it must also have optimized pharmacokinetics, low cost and reproducibility for industrial production. An alternate approach to reduce immunogenicity is to modify it with long polyethylene glycol (PEG) chain that can sterically block the antibodies by masking the protein drug without interfering with its therapeutic applications. PEGylation not only reduces immunogenicity but also solubilize the protein drug by preventing aggregation. Due to the increase in the molecular size, PEGylation can also increase the half-life of the drug enhancing its stability [49, 50]. Hence humanization of our contrast agent and conjugating it with PEG can help us reduce the immunogenicity effects our

1.8. Objective of this Thesis

Initially in this work, previously established first generation of protein-based MRI contrast agent (ProCA1) (designed by senior members Dr. Lixia Wei and Dr. Fan Pu) has been expressed and purified to achieve high yield of ProCA for in vivo GRPR targeting studies. The primary goal of this study is to optimize the production of a humanized third generation of protein-based contrast agent, hProCA32 and to characterize it’s in vitro biophysical properties as a contrast agent. The primary objective is to understand the key determinants of metal binding properties and selectivity over physiological metals. Similarities between rat and human
α-parvalbumin, due to the conserved EF-hand motif, suggests that the functional similarities and relevance of both proteins. hProCA32 can be expected to have similar in vitro and in vivo properties as ProCA32 as an MRI contrast agent. The initial goal is to optimize the expression and purification process using the bacterial system to isolate the pure form of hCA32 with relatively high yield. The secondary goal is to characterize the biophysical and functional properties using fluorescence metal titrations and binding studies. Eventually improve the in vivo properties such as stability, solubility, and relaxivity and reduce immunogenicity and toxicity by modifying hCA32 with various lengths of polyethylene glycol chains (PEGylation). The overall goal is to develop a novel class of protein-based contrast agent with reduced immunogenicity that has similar functional, physiological and contrasting properties as ProCA32.

In Chapter 1 discusses the general background and development of Protein based MRI contrast agent. In Chapter 2, methodologies and experimental details that are implemented for development ProCAs has been described. The bacterial expression, tagless purification and relaxivity measurements of first generation of ProCA and its targeted variants against prostate cancer have been performed in Chapter 3. In Chapter 4, E. coli bacterial expression of hCA32 in various competent cells and its purification will be shown. Furthermore, $^{15}\text{N}$ labeling of hCA32 via E.coli expression and purification has been optimized for future metal binding affinity-using NMR studies (Chapter 4). The site directed mutagenesis of hCA32.cys and its bacterial expression and purification is shown in Chapter 4. Lastly, the structural characterization of hcA32 and its biophysical properties such as relaxivity, binding affinities for terbium, gadolinium, calcium, and zinc, metal selectivity is shown in chapter 5. Site-specific cysteine PEGylation of hCA32 with various lengths of PEG, its stability in human serum and the
enhancing capability of hCA32 has contrast agent is also shown in Chapter 5. Chapter 6 describes the significance of this work and its future applications in the field of MRI imaging and possible targeting against cancer biomarker. The protein-based MRI contrast agents discussed in this thesis are described in Table 1.2.

Table 1.2 Summary of all the ProCAs described in this thesis.

<table>
<thead>
<tr>
<th>Designed contrast agents</th>
<th>Description</th>
<th>Mutations</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProCA1</td>
<td>First generation of ProCA with domain one of rat CD2 as scaffold protein with one Gd$^{3+}$ binding site</td>
<td>N15E, L58D, K64D</td>
<td>11.146 kDa</td>
</tr>
<tr>
<td>ProCA1.G10</td>
<td>ProCA1 grafted with 10 amino acid peptide obtained from GRP</td>
<td>Grafted peptide: GNHWAVGHLM</td>
<td>12.824 kDa</td>
</tr>
<tr>
<td>ProCA1.B10</td>
<td>ProCA1 grafted with 10 amino acid peptide obtained from bombesin</td>
<td>Grafted peptide: GNQWAVGHLM</td>
<td>12.814 kDa</td>
</tr>
<tr>
<td>ProCA1.B14</td>
<td>ProCA1 grafted with full length peptide (14 amino acid) obtained from bombesin</td>
<td>Grafted peptide: EQRLGNQWAVGHLM</td>
<td>13.341 kDa</td>
</tr>
<tr>
<td>ProCA32</td>
<td>The third generation of ProCA with two Gd$^{3+}$ binding sites in a scaffold protein, rat Parvalbumin</td>
<td>S56D, F103W</td>
<td>11.992 kDa</td>
</tr>
<tr>
<td>hProCA32</td>
<td>Humanization of third generation of ProCA with two Gd$^{3+}$ binding sites in a scaffold protein, human Parvalbumin</td>
<td>S56D, D101E, F103W</td>
<td>12.140 kDa</td>
</tr>
<tr>
<td>hProCA32.Cys</td>
<td>hProCA32 with additional cysteine at the C-terminal for site specific PEGylation and labeling</td>
<td>Inserted Cys residue at C-terminal</td>
<td>12.243 kDa</td>
</tr>
</tbody>
</table>

1ProCA1 variants were designed by Dr. Lexia Wei and Dr. Fan Pu. rProCA32 and hProCA32 were designed by Dr. Shenghui Xue.
2. MATERIALS AND METHODS

2.1. Expression of ProCA1 and its variant

2.1.1. Cloning

The DNA sequence of ProCA1 (7E15) was cloned by senior lab member Dr. Anna Wilkins Manicia in accordance with the protocol described in her PhD dissertation. A gadolinium binding pocket was constructed by performing several mutations on a stable scaffold protein, domain 1 of rat CD2. ProCA1.GRP10 (ProCA1.G10) was cloned by inserting codons for 10 amino acid residues from the C-terminal of GRP linked to a flexible linker GGSSGG (Dr. LixiaWei). The DNA sequences of ProCA1.bombesion10 (ProCA1.B10), ProCA1.bombesion14 (ProCA1.B14) and ProCA1.PSMA were cloned by senior PhD student

Figure 2.1 pET-20b(+) vector for ProCA1 variants overexpression.

The DNA sequence of ProCA1 (7E15) was cloned by senior lab member Dr. Anna Wilkins Manicia in accordance with the protocol described in her PhD dissertation. A gadolinium binding pocket was constructed by performing several mutations on a stable scaffold protein, domain 1 of rat CD2. ProCA1.GRP10 (ProCA1.G10) was cloned by inserting codons for 10 amino acid residues from the C-terminal of GRP linked to a flexible linker GGSSGG (Dr. LixiaWei). The DNA sequences of ProCA1.bombesion10 (ProCA1.B10), ProCA1.bombesion14 (ProCA1.B14) and ProCA1.PSMA were cloned by senior PhD student
Fan Pu. All these cloned DNA sequences were inserted into ampicillin resistant pET-20b vector by PCR.

2.1.2. Transformation

Transformation is a commonly used technique to introduce genetic alterations into a host cell through the uptake of foreign DNA. E. Coli strains, BL21 (DE3) were used for transformation and left on ice to thaw for about 10 minutes. Simultaneously, plasmids for ProCA1, ProCA1.G10, ProCA1.B10, ProCA1.B14 and ProCA1.PSMA were also placed on the ice to thaw. A volume of 1uL of plasmid was mixed with 40 μL of thawed BL21 (DE3) competent cells and placed on ice for about 60 minutes. This was done in a sterile environment to avoid any possible contamination. The mixture was then incubated in a heat bath at 42 °C for exactly 90 seconds and then quickly placed on ice for two minutes. Performing this heat shock method is very crucial on the membrane of the E. coli cell strains. A sudden increase in the temperature causes the competent cell to be vulnerable by creating pores on its cell membrane allowing the recombinant DNA to penetrate into the bacterial cells. Approximately 90-100 uL of freshly autoclaved LB medium was added and was further left in an incubator (Fisher Scientific) at 37 °C for 30 minutes. The mixture was then streaked on an agarose/LB ampicillin plate with L-shaped loop and stored in an incubator at 37 °C overnight. The plates were placed upside down for better growth. The following morning each plate was observed for the presence of healthy bacterial colonies. An E.coli bacteria competent cell strain of BL21(DE3) was transformed with the desired plasmids containing pET-20b vector. These plates were covered with parafilm and conserved at 4 °C. This transformation protocol is summarized as a scheme in Figure 2.2
2.1.3. *Inoculation*

In order to effectively express the desired protein, it must be initially overexpressed in a small volume of Luria Bertani (LB) culture medium overnight. A 250 ml flask containing 100 ml LB medium for inoculation was autoclaved at extremely high temperature. A healthy colony from the freshly transformed plate was selected and inoculated in a 250 ml flask of Luria Bertani (LB) medium with 100 mg/L ampicillin. This process was performed under sterile environment to minimize any possible contamination. The inoculated medium was then incubated in a shaker at 37 °C at 230 rpm overnight. The next day, each inoculated flasks were observed for cloudy solution indicating bacterial growth. Using a Uv-Vis Spectrophotometer (Shimadzu UV-Visible), optical density of the inoculated sample was recorded at a fixed wavelength of 600 nm with a final OD$_{600}$ close to 2. These cells were further used to for large-scale expression using *E. coli* cellular system.

2.1.4. *Expression*

A 50 ml volume of overexpressed culture was transferred to one-liter pre-autoclaved LB medium in two-liter flasks containing 100 μg/mL of ampicillin. These flasks were incubated in a shaker at 37°C at 220 rpm. Simultaneously, the rapidly growing bacterial cells were supervised by measuring optical density (OD$_{600}$) using a Uv-Visible spectrophotometer (UV-1601, Shimadzu Scientific). Isopropyl β-D-1-thiogalactopyranoside (IPTG) with final concentration of 200 μM was used to induce the growing bacterial culture, when the optical density reached (OD$_{600}$) reached 0.6-0.8. The protein was continuously expressed at 25°C overnight after the induction with IPTG. The cultured medium was then centrifuged at 7000 rpm for about 30 minutes at 4 °C. Cell pellets were collected and stored at -20 °C. samples were
collected at various time points before and after IPTG induction to confirm the expression of the protein using gel electrophoresis.

Figure 2.2 Summary of Transformation (top) and Expression procedure (Bottom)
2.2. Tagless purification of ProCA1 and its variants

2.2.1. Sonication and cell disruptor

Cell pellet obtained after centrifugation was re-suspended in 20 to 40 ml of homogenization buffer (1X Phosphate-buffered Saline with pH adjusted to 7.4 with HCl). The mixture was mixed with 1.0 uL benzonuclease and 200 uM Phenylmethylsulfonyl-Fluoride, PMSF). Benzonuclease- MgCl₂ is an endonuclease a typical restriction enzyme that cleaves the phosphodiester bonds in a polynucleotide chain and eliminates DNA-RNA form the cell lysate. PMSF is a protease inhibitor that prevents the desired protein from being cleaved by a variety of proteases in the solution and aids in maintaining the purity and yield of the protein. In order to homogenize the mixture, the solution was then mixed well using a vortex. The resulting solution was subjected to sonication 5 times for 30 seconds with 5 minutes interval between each sonication, in order to avoid protein degradation due to heat generated by sound waves of sonicator. The purpose of using sonicator was to break all the bacterial cell membrane by sound waves. The mixture was further homogenized by using a cell disruptor. Like sonicator, cell disruptor is to break the cell membrane and other cellular components from the protein. The mixture was than centrifuged at 17000 rpm for 30 minutes and the supernatant was discarded. Under these conditions, most of the desired protein is contained in insoluble inclusion bodies and the unwanted soluble cellular components were eliminated in the supernatant. The desired protein obtained in the form of pellets were then re-suspended in 2 % Triton, a mild detergent, to wash out unwanted cell debris such as lipid impurities in the form of supernatant. Cell pellets were washed three to four times with 2 % Triton X-100 at 4 °C for about 30 minutes and centrifuged (13000 rpm at 4 °C) between each wash for 30 minutes. After each wash, cell pellets turned white and the supernatant was discarded. Further, the collected cell pellet was
dissolved in 25 ml of 8 M urea prepared in PBS and stirred overnight at 4 °C to completely solubilize the cell pellet. Protein denaturants like 8M urea are quite often used with a purpose of solubilizing the protein subsequently a complete unfolding of the protein, which can then be gradually refolded back. The solubilized protein present in 8M urea solution was centrifuged and supernatant was collected. The obtained supernatant containing soluble and completely unfolded protein was then allowed to gradually refold back by decreasing the Urea concentrations. This was done by dialysis in 4 M urea (overnight) in PBS, followed by 2M urea for 4 hours in PBS. Next, the protein was dialyzed in 10 mM HEPES with pH adjusted to 8.0 with HCl. The buffer was changed after 1 hour, and again after 4 hours, and then allowed to equilibrate overnight. The dialysis was manipulated at 4 °C to prevent the protein from degrading. The obtained sample was then further purified by FPLC using anion exchange column.

2.2.2. Purification by FPLC: anion exchange HiTrap Q Column

Fast Protein Liquid Chromatography (Äkta-FPLC, General Electric, #INV-907, made in Sweden) is a chromatographic process in which the protein solution is exposed to a salt gradient (Q-column, Hi-trap Q-HP5 ml, GE Health care) program. Two buffers were prepared: Buffer A (10 mM HEPES adjusted pH to 8.0 with NaOH) and Buffer B (10 mM HEPES, 1.0 M NaCl at pH 8.0). Buffer A was filtered to remove particles and pH was adjusted. The sample loops were connected with the machine. The anion exchange, Q column and the sample loops were washed using ddH₂O. The Q column and loops were than washed by high salt concentration solution, Buffer B, followed by no salt solution, Buffer A. This was done at flow rate of 5 ml/min, alarm pressure at 5 mPa, for 5 min with no existence of bubbles. A little protein sample was injected
into the sample loops to discharge any air out of the system. Once this was done the entire remaining protein sample was injected into the loop. When the entire sample eluted out into different fractions, they were separated based on the peaks and conserved at -20 °C. The system was washed with high salt concentration buffer and ddH₂O to remove any remaining protein from the system. SDS gel electrophoresis and UV spectroscopy (Shimadzu) were used to check the presence of target protein. The detailed procedure for determining protein concentrations using UV spectrophotometer is described in Section 2.7. UV absorbance at 280nm was monitored to identify the protein from eluted samples, which included protein and DNA. Due to the presence of tryptophan and tyrosine residues, the protein exhibits high absorbance at 280 nm and the nucleotides in DNA strongly absorbs at 260 nm. These results could be rechecked with the SDS gel electrophoresis by comparing the brightness of the protein band at the expected molecular weight of about 12 kDa.
Figure 2.3 Summary of Purification Process for ProCA1 and its variants.
2.3. Expression of humanized ProCA32 (hProCA32)

The DNA sequence of hCA32 was cloned into pET-22b (+) by Dr. Shenghui Xue and the procedure can be obtained from Dr. Shenghui Xue's dissertation[39]. The gene encoding for human alpha parvalbumin with two mutations, one site mutation in the EF hand binding pocket and tryptophan residue close to the binding pocket, were inserted into the pET-22b(+) vector

Figure 2.4 Map of pET-22b(+) vector for hCA32 variants overexpression.

The DNA sequence of hCA32 was cloned into pET-22b (+) by Dr. Shenghui Xue and the procedure can be obtained from Dr. Shenghui Xue's dissertation[39]. The gene encoding for human alpha parvalbumin with two mutations, one site mutation in the EF hand binding pocket and tryptophan residue close to the binding pocket, were inserted into the pET-22b(+) vector.
between the *NdeI* and *Xho I* restriction enzyme sites. Plasmid transformation was performed on pET-22b (+) hCA32 in BL21 (DE3).pLysS and BL21(DE3) cell strains, following the procedure in section 2.1.2. One round colony was selected from a previously transformed plate and inoculated into 200 ml of freshly prepared, and autoclaved LB medium containing 100 ug/mL of ampicilin. The protein was further expressed using the expression procedure mentioned in section 2.1.4

### 2.3.1. Expression of $^{15}$N Labeled hCA32

Plasmid transformation was performed on pET-22b (+) hCA32 in BL21 (DE3).pLysS cell strain following the procedure in Section 2.1.2. One round colony was selected from previously transformed plate and inoculated in 200 ml of freshly prepared M9 medium containing 0.6 g Na$_2$HPO$_4$, 0.3g KH$_2$PO$_4$, 0.15 mg FeSO$_4$, 0.02407 g MgSO$_4$, 0.11 mg CaCl$_2$, 1.0 mL micronutrients, 50mg NaCl, 0.05 mg thiamine, 0.2 g of glucose and 1.0 mL of vitamin supplements and 0.5 g/L of $^{14}$NH$_4$Cl along with 200 uL of ampicilin. $^{15}$N labeled $^{15}$NH$_4$Cl is expensive so the initial small-scale overexpression in 200 mL was done using the $^{14}$NH$_4$Cl. The inoculated flask was left in shaker at 37 °C overnight. The $^{15}$N labeled hCa32 was expressed in M9 medium, which contains 6 g Na$_2$HPO$_4$, 3g KH$_2$PO$_4$, 0.0015 g FeSO$_4$, 0.2407 g MgSO$_4$, 0.0011 g CaCl$_2$, 1.0 mL micronutrients, 0.5 g NaCl, 5 mg thiamine, 2 g of glucose and 10 mL of vitamin supplements. 0.5 g/L of $^{15}$NH$_4$Cl were added as the source for the isotopic nitrogen. One Liter of medium was divided into two 500 mL volumes to get improved yield. Two flasks were left in shaker at 37 °C and OD absorbance were measured during the expression process. Once the OD value reached 0.6, 200 µM of IPTG was added for the induction and the cell pellet was collected after 4-5 hours after induction. The protein was purified using the same purification method mentioned in Section 2.4
2.4. Tagless Purification of humanized ProCA32 (hProCA32)

2.4.1. Sonication and Boiling

In order to obtain protein from the over expressed in \textit{E.coli} cultured medium, the bacterial cells were broken using sonication via application of high energy sound wave to disrupt the phospholipid bilayer and release out the cellular components. For this purpose, the cell pellet obtained from the expression of hCA32 was solubilized in 30 mL in 10 mM HEPES buffer, pH 7.3 along with 1uL of benzonuclease and 1mM PMSF. The mixture was vortexed to generate a homogenous solution. The mixture was then sonicated 10 times with each cycle lasting for 2 minutes. The sonicated solution was further centrifuged at 13000 rpm to collect the supernatant. Collected supernatant was heated at 80-90 °C for about 15 minutes and centrifuged at 13000 rpm. hProCA32 is stable at extreme temperature thus heating will denature unwanted proteins.

2.4.2. Nucleic Acid Precipitation

The DNA and RNA contamination among tagless purification procedure is a common phenomenon and can be resolved using endonucleases. However, the use of benzonuclease alone does not efficiently remove the DNA and RNA when purifying ProCA3 variants. Therefore, in an effort to optimize the purification protocol for all ProCA3 variants, previous lab member, Dr. Shenghui Xue, conducted a series of experiments using streptomycin-sulfate to eliminate nucleic acids from the solution. Streptomycin-sulfate is an antibiotic agent that binds to the DNA/RNA and precipitate then out of the solution. In order to precipitate out DNA and RNA, 3 % of a 30 % streptomycin-sulfate solution was added to the supernatant and left at 4°C overnight. The resulting mixture was centrifuged and supernatant was again heated at 80-90 °C for about 10 minutes and centrifuged at 13000 rpm. The collected supernatant was dialyzed in
10 mM HEPES pH 8.0 at 4°C overnight. The sample obtained from dialysis was centrifuged to remove any possible precipitates formed. The protein was dialyzed in presence of chelax-100 chelating resin to remove as much ions as possible from the protein solution. The supernatant was then passed through FPLC anion exchange Q column to remove DNA/ RNA, and other impurities following the protocol mentioned in section 2.2.2.

2.5. Molecular Cloning

2.5.1. Site directed Mutagenesis for humanized ProCA32.Cys

In order to insert a cysteine residue at C-terminus of hCA32, a general procedure for site-directed mutagenesis was performed. A cysteine residue was inserted into the plasmid by forward and reverse primer using polymerase chain reaction (PCR). Both forward and reverse primers were phosphorylated prior to the experiment. Annealing temperature is the key factor for successful PCR. Thus keeping the length of the primers and the template DNA in mind, $T_m$ was kept at 65 °C for the total time of 6 minutes. The PCR product was obtained and digest with $DpnI$ restriction enzyme and incubated at 37° C for about 2 hours and kept at 4° C overnight. Further the product was transformed using XL10 gold competent cells and grown on LB/ampicillin plate. XL10 gold competent cells are often used for amplification for cloning and ligated plasmid to obtain high transformation efficiencies. About eight round and healthy colonies were selected for inoculation into 10 ml of LB medium and left on shaker at 37° C overnight. The DNA was extracted using the general amplification protocol. The amplified plasmid was then digested using $Xho I$ cleavage site to cut open the circular plasmid for further analysis using an agarose gel electrophoresis. The correct plasmid constructs were confirmed by DNA sequencing.
2.5.2. Plasmid reconstruction of pRSET-CD20

2.6. Expression and purification of hCa32.cys

Plasmid transformation was performed on pET-22b(+) hCA32.cys in BL21(DE3).pLysS E.coli cell strain following the procedure in 2.1.2. One round colony was selected from previously transformed plate and inoculated in 200 ml of freshly prepared and autoclaved LB medium along with 200 uL of ampicilin. The Protein was further expressed using the expression procedure mentioned in section 2.1.4 and purified using the protocol mentioned in section 2.3

2.7. Concentration Determination using Ultraviolet-visible absorbance spectra

The concentrations of purified protein solutions were determined using a UV-VIS spectrophotometer by monitoring UV absorbance ranging from 350-219 nm (UV- probe version 2, Pharma spec. 1700, Shimadzu scientific Instrumentation Inc. Newton, CT). Quantitatively, the UV-VIS absorbances were recorded for each concentrated protein solution, in order to experimentally determine the final protein concentration (C). Two quartz cells with a length of 1 cm containing 800 μL of nanopure water (ddH₂O) was scanned from 350-219 nm to create a baseline in order to subtract the absorbance differences. One of the quartz cells was replaced with a cell containing protein solution and measured from 350-219 nm. The detection limits for UV absorbance ranges from 0.1 to 1.0. Hence, the protein solutions were diluted if their absorbances exceeded 1.0. The absorbance maximum value at 280 nm displayed in the spectrum was multiplied by the dilution factor to obtain C in the Equation 2.1. The molar absorptivity (ε) of wild-type CD2.domain1 was experimentally determined by previous lab members, based on the presence of tryptophan, tyrosine and disulfide bonds[51]. The theoretical value obtained based on primary amino acid sequence using online database Protein calculator. (Available at http://www.basic.northwestern.edu/bio tools/proteincalc.html). The experimental
molar absorptivity of ProCA1 is 11740 M⁻¹cm⁻¹ where as the theoretical molar absorptivity estimated by protein calculator, 13940 M⁻¹cm⁻¹. The theoretical extinction coefficients of ProCA1 variants (ProCA1.G10, ProCA1.B14 and ProCA1.B10) are 19630 M⁻¹cm⁻¹.

The theoretical molar absorptivity of hCA32 variants determined using online data base protein calculator was 5690 M⁻¹cm⁻¹. The experimental molar absorptivity value was used in the Beer Lambert equation for obtaining $C_F$

$$A = \varepsilon bC$$  \hspace{2cm} \text{Equation 2-1}$$

2.8. Western Blotting

Gel electrophoresis with 15% percent SDS gel was used to run the protein samples. The electrophoresed gel was placed in a Blot Module with the nitrocellulose transfer membrane. The electrophoresed gel and membrane are sandwiched between sponge and filter paper and all are clamped tightly together after ensuring no air bubbles are trapped between the gel and membrane. The negatively charged proteins in the electrophoresed gel are transferred onto the nitrocellulose membrane under 225 mA for about 2 hours in transfer buffer (25 mM Tris-HCl, 190 mM glycine and 20 % methanol). Blocking the membrane prevents non-specific background binding of the primary and secondary antibodies to the membrane. Hence, the nitrocellulose membrane was placed into the blocking solution (filtered 5% BSA in TBST, Tris Buffer Saline Tween20 buffer) for 40 minutes at room temperature. The membrane was then incubated with primary antibody (mouse anti-hCA32 primary antibody) diluted with 1:1000 dilution ratio in freshly prepared blocking buffer at 4°C overnight. The next day, membrane was washed using TBST buffer 3-4 times each time lasting 5 minutes at room temperature. The secondary antibody (anti-mouse secondary antibody, AP conjugated), diluted in fresh blocking
buffer with 1:10,000 dilution ratio was applied to the membrane and incubated at room temperature for about an hour. The membrane was washed in TBST 3 times and each time lasts 5 minutes. The location of the antibody can be revealed by using a colorless substrate that enhances once it binds to the secondary antibody and further be seen and photographed. One ml of substrate along with 25 uL of enhancer was added to the membrane and a photo was transferred on a film.

2.9. Relaxivity measurements

The concentrated protein samples were first allowed to dialyze and chelaxed using 10mM HEPES-Chelaxed buffer at pH7.3 for 48 hours to remove free metal ions from the protein solution. Relaxivity measurements were taken using fixed concentration of Gd$^{+3}$ and increasing protein concentration. The T$_1$ and T$_2$ relaxation time of ProCA1 variants and hCA32 were measured by a Bruker MiniSpec relaxometer. The T$_1$ is the time of longitudinal magnetization recovery and T$_2$ is the time of transverse magnetization recovery to equilibrium along the direction magnetization. 200 ul of the solution containing varying concentrations of contrast agents, fixed [Gd$^{+3}$] and 10 mM HEPES pH 7.3 was added to a clean glass tube specially used for this instrument. Glass tubes were incubated in the instrument until it reached to equilibrium state and T1 and T2 values were measured. R1 and R2 were calculated using

Equation 2-2

\[
R_{1,2} = \left( \frac{1}{T_{1,2} \text{Sample}} - \frac{1}{T_{1,2} \text{Buffer}} \right) \left[ \text{Gd}^{3+} \right]
\]

Equation 2-2
2.10. **Terbium Titration using Metal-Chelator Buffer System**

All the acquired fluorescence spectra were recorded using a fluorescence spectrophotometer (Photon Technology International, Inc.) with xenon short arc lamp at ambient temperature. Luminescence resonance energy transfer (LRET) experiments for Tb$^{3+}$ were acquired by tryptophan excitation at 280 nm and emission from 500 to 650 nm. For the Tb$^{3+}$-EGTA or Tb$^{3+}$-DTPA buffer system 30 μM of hCA32 were added into the buffer containing 50 mM HEPES, 150 mM NaCl, 5mM EGTA or DTPA at pH 7.2 and 0.05 uM of Rhodamin-5N, tripotassium salt (Invitrogen Molecular Probes). Rhod-5N, tripotassium salt is a weak calcium indicator, which can detect the presence of any free Ca$^{2+}$ with a binding affinity of 320 μM[52]. As mentioned earlier, trivalent terbium is known to selectively bind to calcium binding sites hence calcium-binding dyes are often used to mimic similar conditions for determining Tb$^{3+}$ affinities. Since rhodamin-5N has lower binding affinity for metal, throughout the titration the metal is either bound to chelator (EGTA or DTPA) or the protein. Rhodamin-5N dye was used as an internal standard to detect the chelator (EGTA or DTPA) and protein saturation caused by Tb$^{3+}$ in the buffer system ($\lambda_{ex}= 551$ nm, $\lambda_{em}= 576$ nm). Then, TbCl$_3$ was titrated into the buffer with protein. The concentrations of Free Tb$^{3+}$ were calculated by Equation 2-3. The protein is excited at 280 nm so the tryptophan residue near the binding pocket is excited and transfers energy to the bound metal Tb$^{3+}$, which further has a fluorescence emission at 545 nm. As the Tb$^{3+}$ concentration increases, an increase in fluorescence intensity must be observed due to the emission of Tb$^{3+}$ bound in the vicinity of tryptophan residue.

$$\left[ Tb^{3+} \right]_{free} = K_d_{Tb-DTPA} \times \frac{[Tb-DTPA]}{[DTPA]_{free}} \quad \text{Equation 2-3}$$
\[ f = \frac{[Tb^{3+}]_{\text{free}}^n}{[Kd]_{Tb-DTPA}^n + [Tb^{3+}]_{\text{free}}^n} \]  

**Equation 2-4**

*Note: The same equations were analogously used for the buffer system with EGTA as a chelator.*

\([Tb^{3+}]_{\text{free}}\) is the free Tb\(^{3+}\) concentration calculated from buffer system, \(K_d\) \(\text{Tb, EGTA or DTPA}\) is the dissociation constant of Tb\(^{3+}\) and EGTA or Tb\(^{3+}\) and DTPA, the dissociation constants of Tb\(^{3+}\) to EGTA \((10^{-18}M)\) and DTPA \((10^{-22}M)\) were obtained from NIST. \([Tb-DTPA]\) is the concentration of Tb-DTPA complex that is formed during titrations, \([DTPA]_{\text{free}}\) is the free DTPA in the buffer, \(f\) is the fractional change, \(K_{d,Tb,hCA32}\) is the dissociation constant between Tb\(^{3+}\) and hCA32.

**2.11. Determining Metal Binding Affinity: Gd\(^{3+}\), Zn\(^{2+}\), Ca\(^{2+}\), Lu\(^{3+}\)**

All the acquired fluorescence spectra were recorded using a fluorescence spectrophotometer (Photon Technology International, Inc.) with xenon short arc lamp at ambient temperature. As gadolinium does not fluoresce, terbium fluorescence was utilized via LRET competition method to measure the Gd\(^{3+}\) binding affinity to hCA32. The terbium fluorescence spectra were acquired by tryptophan excitation at 280 nm and emission from 500 to 650 nm. 10 \(\mu\)M of hCA32 and 20 \(\mu\)M Tb\(^{3+}\) were incubated with 0 to 1000 \(\mu\)M of GdCl\(_3\) at room temperature over night. The Tb\(^{3+}\) FRET changes were measured by the emission at 545 nm. The apparent \(K_d\)'s were fitted by 1 to 1 binding Equation and \(K_d\) of Gd\(^{3+}\) were calculated by **Equation 2-5** and **Equation 2-6** and the \(K_d\) \(hProCA32-Gd\(^{3+}\) was calculated using **Equation2-7**
\[ \Delta f = \frac{(P_T + M_T + K_d) - \sqrt{(P_T + M_T + K_d)^2 - 4P_T M_T}}{2P_T} \]  
Equation 2-5

\[ \Delta f = \frac{1 - (M_2 + M_0 + M_1) - \sqrt{(M_2 + M_0 + M_1)^2 - 4M_2 M_0}}{2M_2} \]  
Equation 2-6

\[ K_{hProCA32-Gd} = K_{app} \frac{K_{d1}}{K_{d1} + [Tb^{3+}]_{Total}} \]  
Equation 2-7

Gadolinium binding of hCA32 was also determined by competition titration using Fluo5N dye. Fluo-5N (Invitrogen Molecular Probes) is used as gadolinium indicator with a \( K_d \) of \( 3.8 \times 10^{-12} \text{ M} \) [29]. The Fluo-5N emission spectra were obtained by excitation at 488 nm and emission from 500-650 nm. Fluo-5N and Gd\(^{3+}\) were mixed with 1:1 (2-4 \( \mu \text{M} \)) ratio in 10 mM HEPES at pH 7.0 and Protein was gradually titrated into the cuvette. As protein concentration increases in the system, the protein competes with the dye causing a decrease in the fluorescence signal. The signal decreases until the protein is saturated. The fluorescence intensity decrease of Fluo-5N at 520 nm with titration of various hCA32 concentrations were fitted using one to one binding equation, Equation 2-5 to get an apparent dissociation constant \( K_{app} \). The Gd\(^{3+}\) binding affinity of hCA32 was calculated using Equation 2-8 where \( K_{d1} \) is the binding affinity of Fluo-5N and Gd\(^{3+}\).

\[ K_{hProCA32-Gd} = K_{app} \frac{K_{d1}}{K_{d1} + [Fluo-5N]_{Total}} \]  
Equation 2-8

The Zinc binding affinity of ProCA1 variants and hCA32 were determined using a similar assay by competition titration using FluoZin1 dye (Invitrogen Molecular Probes). The
binding affinity of contrast agents for zinc must be comparatively low, as zinc is present in physiological conditions, which can hinder the formation of ProCA-Gd$^{3+}$ complex may affects the relaxation rate and MRI signals. In order to obtain the binding affinities of Zn$^{2+}$, various concentrations of contrast agents were gradually titrated into the 1:1 dye-metal complex (FluZn-1-Zn$^{2+}$) to compete for the Zn$^{2+}$ ions bound to the dye. Fluorescence emission spectra of FluOZin1 were obtained from 500-600 nm with an excitation at 495 nm and decrease in intensities was monitored at 515 nm. Apparent $K_d$ was obtained by fitting the data using one to one binding equation, **Equation 2-5**. The following equation was used to calculate the $K_d$ value of hCA32-Zn binding where $k_{d1}$ is the binding affinity of FluOZin1 and Zn$^{2+}$ (2.2×10$^{-12}$ M) [29].

$$K_{hProCA32-Zn} = K_{app} \frac{K_{d1}}{K_{d1} + [FluoZin-1]_{Total}}$$  \text{Equation 2-9}

For determining the calcium binding affinity of hCA32, calcium equilibrium buffer system was used where, 5 μM hCA32 was added to the calcium –EGTA buffer system containing 50 mM HEPES, 150 mM NaCl, 5 mM EGTA, pH 7.2. Various calcium chloride concentrations were titrated to the buffer system. Free calcium concentrations in the buffer were tightly monitored and calculated using equation derived from tsein’s equation (**Equation 2-10**) where $K_d$ of EGTA-Ca$^{2+}$ = 1.51x10$^{-7}$ M was obtained from NIST[53].

$$[Ca^{2+}]_{free} = K_{d, Ca-EGTA} \times \frac{[Ca-EGTA]}{[EGTA]_{free}}$$  \text{Equation 2-10}

### 2.12. PEGylation

To optimize the protein solubility, blood circulation time, avoid possible enzymatic degradation and reduction of immunogenicity; Poly ethylene glycol (PEG) conjugation with
protein has been often used in pharmaceutical and biotechnical applications. The desired protein can be modified using several PEG conjugation strategies such as amino group modification, thiol group modification. hCA32 has been PEGylated with NHS-Ester on lysine residue and PEG-Maleimide on cysteine residue. The detailed procedures are in the following sub section.

2.12.1. Lys-PEG

The contrast agent was modified using Methyl-POE₃₋₄-NHS ester, PEG-40 (Thermo Scientific Pierce) with molecular weight of 2.34 kDa and 40 repeating units of ethylene glycol. At first, hCA32 and PEG-40 (Pierce) were mixed with 1:5 ratios in 10 mM HEPES buffer (pH 7.2) and allowed to shake at room temperature for about 2 hours or at 4 °C overnight. Further, 100 mM Tris/HCL was added to the reaction system in order to stop the reaction by quenching the unreacted free PEG-NHS ester. The mixture was then concentrated about 4 times using amicon nitrogen concentrator with 3kDa membrane to remove the unreacted free PEG (MW 2.34 kDa). The PEGylated protein was identified by Iodine staining and SDS-PAGE. Iodine can stain the free PEGylated protein along with free PEG in the solution while Coomassie brilliant blue can stain the protein that is already conjugated with PEG.

2.12.2. Cys- PEG

The PEGylation of free and unessential cysteine residues in the protein is another approach that is gaining popularity. Majority of protein naturally contains multiple lysine residues as a result multiple isomers can be formed when modified with lys-PEG. In order to avoid such complications, PEG modification at cysteine can be used to attain homogeneity. If a primary amino acid sequence of a protein lacks cysteine residue, it can be easily introduced via genetic engineering and molecular cloning. At first, the protein with concentration of about 1-10 mg/ml was dissolved in 10 mM HEPES buffer, pH 7.2. This solution was flushed with inert gas
such as nitrogen, argon or helium gas. Access of TCEP (Tris-carboxyethylphosphine) reagent (Thermo Scientific Pierce), 1:10 volume ration was added to reduce the disulfide bonds in the presence of inert gas. This mixture was left on a shaker for 20 minutes at room temperature. Methyl-PEG Maleimide reagent (Thermo Scientific Pierce and JenKem Technology) with molecular weights of 0.710 kDa, 1.2 kDa, 2kDa and 5kDa were added to the buffered hCA32 mixture with a ratio of 1:5 in the presence of inert gas and the tube was closed tightly to avoid the escaping of gas. The solution was left shaking overnight at 4 °C.

**2.13. Serum Stability**

The serum stability test is one of the useful tests to confirm the stability of an unstable biological compound *in vitro*. The serum stability of 2kDA-PEGylated and non-PEGylated hCA32 was analyzed by incubating the designed protein in a complex form with an equal concentration of GdCl₃ in mice serum at 37 °C. hProCA32-Gd³⁺ (500 μM) was mixed with mice serum with 1:1 ratio and incubated at 37 °C for 12 days. During this time frame 10 μL of samples were collected at various time points. These samples were analyzed by SDS-PAGE and western blot to confirm the stability of the protein.
3. DEVELOPMENT OF RAT PROCA1 AND ITS ENGINEERED VARIANTS VIA GRAFTING APPROACHES

3.1. Introduction

As discussed in Chapter 1 (Section 1.7), Dr. Yang’s Lab has developed a series of protein-based molecular imaging contrast agents by constructing a metal binding pocket, which in our case is for gadolinium. The scaffold protein, domain 1 of rat CD2 was selected by keeping its physical characteristic such as stability, solubility and molecular size into consideration. The first generation of protein-based MRI contrast agent (ProCA1), developed and analyzed by previous lab members, has high \( r_1 \) and \( r_2 \) relaxivities compared with the clinical contrast agents both in vitro and in vivo[29]. In order to enhance the diagnostic capabilities of our contrast agent for the early detection of cancer, ProCA1 was targeted with affibody that can recognize the biomarkers EGFR and HER2 in breast cancer cells [10]. In our approach to broaden the horizon of the capability of ProCA1 to target the diseased biomarkers; we have further modified the contrast agent with GRP to target the receptor GRPR, which is highly expressed on prostate cancer cells. Initially, ProCA1 was modified in two different ways: (1) by adding GRP at C-terminal (ProCA1.GRPC), and (2) by grafting GRP between Gly52 and Gly53 (ProCA1.GRP (52)) in the protein sequence. Amongst these two variants, ProCA1.GRP (52) showed higher metal binding affinity for \( \text{Gd}^{3+} \) and stronger \( \textit{in vivo} \) biomarker targeting capability as compared to ProCA1.GRPC with detection of GRPR expressed on, as low as \( 10^7 \) cancer cells (tumor size of < 0.3 cm)[27]. So to improve the GRPR targeting capability of ProCA1, various lengths of GRP peptides native to mammalian cells or bombesin peptide, found in amphibians were grafted into ProCA1. In order to move further with the \( \textit{in vivo} \)}
studies, the production process of the contrast agents must be optimized to yield high quality, stable and well-folded ProCAs.

In this chapter, the bacterial *E.coli* expression in LB medium and tagless purification process of targeted ProCA1 variants namely ProCA1.G10, ProCA1.B10 and ProCA1.B14 are described. Further, the relaxivity studies and zinc binding affinities of these variants are presented.

### 3.1. Results

#### 3.1.1. Expression of ProCA1 and its variants

ProCA1 and its variants were cloned into a tagless vector pET20b (+) by previous lab members, as mentioned earlier in the section 2.1.1. These variants were first expressed at a small-scale level in 10 mL of LB medium to optimize the expression conditions. Small Scale expression is usually performed to attain higher yield by establishing suitable conditions. Another important purpose of doing small-scale expression is to monitor the expression of ProCA1 in two *E.coli* cell strains; BL21 (DE3) and Tuner. Initially three clones were selected from agar plates with ProCA1 cloned into BL21 (DE3) and Tuner. These selected colonies were expressed using the protocol described in sections 2.1.3 and 2.1.4. The overexpression of ProCA1 in these colonies is shown in the Figure 3.1A and Figure 3.1B. A typical process for bacterial growth of *E.coli* cells has four stages, namely, lag phase, log phase, stationary phase and death phase. The cellular growth curve shows an exponential increase with time in minutes (Figure 3.1 C and D). In SDS-PAGE gel (Figure 3.1A) a slight band at ~ 11 kDa in the samples for before-induction (lanes 1 and 3) indicates the occurrence of leaky expression in BL21DE3 cell strain. However, a bright band at 11 kDa is observed for all the clones picked
(lanes 2, 4 and 6) after the IPTG induction and temperature reduction to 25°C. Similar phenomenon was observed for protein expressed in Tuner cell strain, colonies 1 and 2 (lanes 1-4) (Figure 3.1 B). Tuner cell strain has a tendency to produce inclusion bodies, which acclimates the expressed protein and sediment with the pellet. Intense bands around 25 kDa in SDS PAGE gel indicates that along with the desired protein ProCA1, other unwanted proteins were also expressed in higher quantity as compared to ProCA1 (11kDa). Large-scale expression was performed using the remaining samples of the selected cell strains. Although the optical density curve for BL21 (DE3) at 37 °C was exponential, a low level expression was seen after the induction with 1 mM IPTG induction. Thus, reducing the temperature did not have any significant effect on the expression level. This could have happened either due to bad quality of the cloned selected or contamination of the growth medium.
Figure 3.1 Small scale expression of ProCA1 in various E.coli cell strains

A) Small scale expression gel of ProCA1 in Bl21DE3 cell strain, where M is Marker with standard molecular weight, 1, 3 and 5 are before induction and 2,4,6 are after induction for colonies a, b and c respectively. B) Small scale expression gel of ProCA1 in Tuner cell strain, where M is Marker, 1, 3 and 5 are before induction and 2,4,6 are after induction for colonies a, b and c respectively. C) Cellular growth plot at various time periods during the expression of ProCA1 in BL21DE3 cell strain. D) Optical density during the cellular growth at various time periods during the expression of ProCA1 in Tuner cell strain.

*Note: All the samples added to gel electrophoresis are of equal volume of the culture.
Subsequently, Large-scale expression of ProCA1 was performed using the aforementioned protocol in BL21 (DE3) and Tuner *E.coli* cell strains in LB medium. The expression level of ProCA1 in BL21 (DE3) cell strain was optimized by various IPTG concentrations administered at induction. The exponential increase in optical density to the point of induction for ProCA1 in BL21 (DE3) indicates a bacterial cell growth throughout the expression process (Figure 3.A). A distinct band is observed at around 11 kDa prior to induction indicates the occurrence of a residual “leaky” expression in BL21 (DE3) (Figure 3.B). Such leaky expression is caused due to initiation of transcription in the absence of inducer and is also known as basal expression [54]. There are many possible reasons that may lead to leaky expression; the Lac repressor protein does not bind to the DNA efficiently and remains unable to shut off the gene transcription from the lac operator promoter. The expression cloning constructs such as pET, pUC etc. are often made to generate high copy number of plasmid which may further lead to the potentially increase in the copy number of promoter and operator causing leaky or basal expression [54, 55]. At induction, the cells received various concentrations of IPTG, 0.1 mM, 0.3 mM, 0.5 mM and 1.0 mM and were allowed to express at 30 °C overnight until harvesting. All the IPTG concentrations seemed to have similar effect on the expression as thick bands are observed after the overnight expression at 30 °C (Figure 3.2B).

In case of large-scale expression of ProCA1 in Tuner cell strains, their expression level was comparable to that of samples expressed in BL21 (DE3) Tuner cell strain is a mutant of BL21 with a deletion of lacZY, a mutation of lac permease (lacY). Such mutation allows the uniform entry of an inducer, which in our case is IPTG into the cell throughout the cultured medium making the induction in a true concentration dependent fashion [56]. Hence, the
expression can be regulated and improved by increasing the inducer, IPTG concentrations. Instead of testing the effects of various concentrations of IPTG as in case of BL21 (DE3) cell strains, maximum amount of IPTG (1mM) was used for the large-scale protein expression in Tuner cell strains. The optical density curve increased exponentially to the point of induction indicating a fairly well bacterial growth (Figure 3.2C). A faint band at 11 kDa is observed in SDS-PAGE gel suggesting the occurrence of leaky expression (Figure 3.2D). High optical density value after the overnight expression at 30 °C indicates that Tuner cells produced a large amount of inclusion bodies with a net pellet weight of 5.91 g/L. However, thick bands 25 kDa to 60 kDa in SDS-PAGE gel suggests that a large amount of unknown proteins were co-expressed along with the desired protein.

All the targeted ProCA1 variants, ProCA1.G10, ProCA1.B10, and ProCA1.B14 were all overexpressed in BL21 (DE3) competent cells. As seen in Figure 3.3, all the variants seem to have clear bands at 12 kDa in SDS-PAGE gel both before and after 1 mM IPTG induction. ProCA1.B14 has a cysteine in the targeting peptide grafted on ProCA1, which can possibly form a disulfide bond resulting into dimerization of the protein expressed. Hence, a distinct, bright band is observed at ~ 24 kDa in SDS-PAGE gel (Figure 3.3C) both before and after induction where the SDS sample buffer did not contain any reducing reagent.
Figure 3.2 Large scale Expression of ProCA1 in *E. coli* cell strains using LB medium.

((A) Cellular growth curve and (B) 15 % SDS-PAGE of ProCA1 expressed in BL 21(DE3) with before and after induction where various IPTG concentrations were used for the samples for after induction (0.1mM, 0.3 mM 0.5 mM and 1 mM). (C) Cellular growth curve (D) 15 % SDS-PAGE of ProCA1 expressed in Tuner.

*Note: All the samples added to gel electrophoresis are of equal volume of the culture.*
Before induction and after induction by 1mM IPTG has evidenced difference in the band intensity at ~12 kDa. (C) For ProCA1.B14, dimer form of the protein is observed at ~24 kDa. The SDS sample buffer did not contain any reducing reagent and the SDS gel was a non-reducing gel.

*Note: All the samples added to gel electrophoresis are of equal volume.
3.1.2. Tagless purification by denaturing and refolding method

ProCA1 variants expressed in LB medium were purified using the procedure described in Figure 2.3 (Section 2.2). After harvesting the cell pellet, it was suspended in 30 mL of PBS for cell lysis. Subsequently, the homogenized cell lysis was achieved by the use of sonication followed by cell disruptor. The inclusion bodies formed during the expression were found in the pellet after breaking the cells (Figure 3.5 A). The purification process for ProCA1 expressed using BL21 (DE3) competent cells illustrated in Figure 3.5. During the rapid expression of the protein, large amounts of cellular components and broken cell membranes are expressed which, after breaking of cell membrane, are released into the cell lysis solution and remain in the pellet along with the protein after centrifugation. Detergents are widely used to isolate the protein from cell membrane as stabilizing and denaturing reagents [57]. Triton x100 is often used for solubilizing the lipid bilayer in the cell lysis by interacting with the polar group present on the hydrogen-bonding region. In our case, Triton x100 can be used to solubilize the insoluble lipids bilayer that coexists along with the desired protein in the pellet. In order to get rid these lipids and insoluble waste, the pellet is re-suspended three times into 2% of Triton 100 X where the protein remains insoluble in the pellet. Due to the presence of large amount of hydrophobic lipids in the protein solution, the clarity of the protein band in SDS gel can be affected. This clarity of the protein band in pellet seems to improve after the 2nd triton application. SDS-PAGE gel (Figure 3.5 A) shows a bright band at ~11 kDa (in the pellet), indicating that majority of unwanted materials and proteins were indeed solubilized and discarded into the supernatant while the desired contrast agent remained in the pellet. Some of the ProCA1 seemed to be eliminated along with the waste, which might have been due to following reasons: 1) Triton 100 X could be a little harsh for the protein, if the protein solution is washed for too long the
detergent may denature it. 2) The solution might not be homogeneous prior to the centrifugation that may lead to improper separation of the pellet and supernatant 3) Error in preparing the SDS sample where the samples were not centrifuged properly to separate completely the supernatant from the pellet. Again due to rapid expression as inclusion bodies, a lot of protein molecules may have misfolded. Thus, the precipitates obtained were allowed to solubilize in 8 M urea causing the protein to unfold completely from its native folding. The soluble protein was separated in the supernatant from the insoluble waste. The next step was the gradual refolding of the protein by dialysis in reducing urea concentration from 4 M urea to 2 M urea followed by 0 M urea (10 mM Tris at pH8.0). As seen SDS-PAGE gel (Figure 3.5B), the lone high-density band is seen at 11 kDa after dialysis suggesting that the majority of the solution consisted of ProCA1. The next step is to remove any possible small fragments of waste and DNA using anion exchange chromatography. The Äkta-FPLC system and Hi-Trap Q column packed with sepharose are washed and recharged using the procedure described in section 2.2.2. After washing the column with high salt containing buffer B (10 mM HEPES, 1.0 M NaCl at pH 8.0) followed by buffer A (10mM HEPES at pH 8.0), protein solution filtered with 0.45 mm Millipore membrane filter was injected into the system. The first step is the binding step where the negatively charged residues on ProCA1 bound to the positively charged column for 5 column volumes (CV). The salt gradient was introduced to 35 % for 1 CV followed by a gradual increase to 40 % over another 2 CV. The salt gradient was held at 40 % for next 2 CV until it reached to 100 % buffer B. The protein had negatively charged residues but the total negative charge was not as much as nucleic acids. Hence, the protein was expected to elute at lower salt content.
As seen in the FPLC chromatogram of ProCA1 expressed in BL21 (DE3) (Figure 3.5C), four peaks were observed. SDS-PAGE gel of the fractions indicated that majority of the ProCA1 indeed eluted out as the salt concentration increased, under peak 2. Peak 3 and 4 are not well separated as seen in the spectrum; however, the SDS gel demonstrates that both peak 3 and 4 contains our desired protein (Figure 3.5 B). Since the anion exchange chromatography separates each component based on its charge, it can be concluded that elution under peak 3 and 4 are more negatively charged as compared to the elution under peak 2, which might be due to DNA contamination. Hence, although the SDS-PAGE gel is indicative of the presence of the protein in peak 3 and 4, it is obvious that both the peaks contain a mixture of protein and DNA. Some of the protein also elutes out under peak 3 (Figure 3.5 B). Therefore, ProCA1 expressed in BL21 (DE3) cells was extracted in its pure form under peak 2 using tagless purification and anion exchange chromatography. Another batch of ProCA1 was expressed in BL21 (DE3) and purified. The FPLC spectrum (top) and the 15 SDS gel (bottom) of Figure 3.6 shows that protein eluted under peak 2.
Figure 3.5Tageless Purification of ProCA1 expressed in BL21 (DE3) competent cells by Refolding method

(A) 15 % SDS PAGE: The first step of the purification by washing the cell pellet in 2 % Triton 100 X. The protein band brightens by the third wash. (B) 15 % SDS-PAGE: The second step of purification by denaturing the protein to make it soluble along with removing other unwanted waste that is insoluble or denatured in the cell pellet. Gradually refolding the protein by dialyzing it in reducing urea concentration of the buffer. (C) The final step of the purification by anion exchange Q- column using FPLC to eliminate small molecular weight, unwanted components that co-exists in the solution.
Figure 3.6 Purification by FPLC of another batch of ProCA1 expressed in BL21 (DE3) competent cells

The FPLC spectrum shows 3 major peaks where the protein eluted out under peak 2. Peak 2 was divided into two separate portions: 2a-fractions under high Uv absorbance and 2b) fractions under the tailing part of the peak as it may contain DNA in it. The 15 % SDS gel confirms the presence of ProCA1 under peak 2a.
Three major peaks are observed in the FPLC spectrum for ProCA1 expressed in Tuner cell strains. In comparison with ProCA1 expressed in BL21 (DE3) cells, Tuner cells had a large amount of DNA expressed in the inclusion bodies. If the protein solution, prior to anion exchange chromatography contains large amount of DNA/RNA or nucleic acid fragments that have more negative charge as compared to protein (ProCA1), it may compete with the protein during the binding process hindering the protein to bind to the column. So due to the presence of large amount of nucleic acids in the protein solution expressed in Tuner cells, the protein was unable to bind with the column as result high UV absorbance is observed during the binding step of the FPLC program (Figure 3.7 C). Furthermore, the UV spectrum of elution under peak 1 has high absorbance at 280 nm whereas the elution under peak 2 has high absorbance at 260 nm suggesting that peak 1 had majority of protein compared to peak 2 (Figure 3.7 B), which is supported by the presence of band at 11 kDa in SDS-PAGE gel (Figure 3.7 A). A slight shoulder in Peak 2 is observed in the FPLC spectrum, which suggests that the fractions under the shoulder may contain some DNA.

The GRPR targeted ProCA1 variants (ProCA1.G10, ProCA1.B10 and ProCA1.B14) inserted with various targeting moieties were expressed in BL21 (DE3) and purified using tagless purification protocol mentioned in Figure 2.3 (Section 2.2). The purification results for each variant are shown in the Figure 3.8-3.10
Figure 3.7 The tagless purification of ProCA1 expressed in Tuner cell strain by refolding method.

(A) SDS-PAGE gel after solubilizing it in 8 M urea and refolding it gradually by dialysis in reducing urea concentrations. (B) UV spectra of peak 1 and peak 2 separated by FPLC. (C) The FPLC chromatogram of ProCA1 expressed in Tuner.
Figure 3.8 15% SDS PAGE depicting the purification steps of ProCA1.G10

(A) The first and second step of purification by 3 times 2% Triton 100 X and unfolding of the cell pellet by 8 M Urea. Majority of the unwanted protein at 26 kDa still coexisted prior to FPLC injection. (B) The SDS-PAGE of the fractions under FPLC spectrum where majority of the protein was isolated under peak 2 and peak 3.
ProCA1.B14

A) 15% SDS- PAGE of the first and second step of purification by 3 times 2% Triton 100X and unfolding of the pellet by 8 M Urea. Some of the unwanted proteins were removed in the pellet separated after dialysis suggesting that exposure to 8M urea was too harsh causing them to degrade or the proteins were unable to refold back after being denatured by 8M Urea. However, the presence of SDS bands between 14 kDa and 26 kDa and at 68 kDa indicates that some wanted protein were indeed refolded back along with ProCA1.B14.

B) The SDS-PAGE of the fractions from FPLC spectrum shows that the majority of the protein was isolated under peak 2 and peak 3.

Figure 3.9 15% SDS PAGE depicting the purification steps of ProCA1.B14 expressed in BL21(DE3) cell strain

C) Peak 2 Protein

Peak 3 Protein/DNA

Peak 4: DNA
Figure 3.10 15 % SDS-PAGE analysis of the purification process of ProCA1.B10 expressed in BL21(DE3) E.coli cell strain.

A) 15 % SDS- PAGE of the samples after sonication shows that ProCA1.B10 remained in pellet (band at 12 kDa) and first wash with 2 % Triton 100 X removed some unwanted proteins (bands at 55 kDa). (B) The band at 12 kDa are enhanced and brightened (in the pellet) due to the removal of lipids by the remaining two Triton wash. After unfolding of the protein solution by 8 M urea the band intensity at 25 kDa reduced suggesting that those protein were unable to fold back after being denatured. (B) The FPLC fractions under peak 2 were separated as The SDS-PAGE of the fractions from FPLC spectrum shows that the majority of the protein was isolated under peak 2a.
Figure 3.11 Western Blot and 15 % SDS-PAGE of purified ProCA1 variants.

(A) Western blot of the concentrated samples of ProCA1 variants identified by anti hCA32 primary antibody. (B) 15 % SDS PAGE of the concentrated samples of ProCA1 variants.

Table 3.1 Final yields of the ProCA1 variants purified using tagless refolding method.

<table>
<thead>
<tr>
<th></th>
<th>Final Volume (ml)</th>
<th>Final concentration (μM)</th>
<th>Final yield (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProCA1.B14</td>
<td>5.00</td>
<td>94.87</td>
<td>6.33</td>
</tr>
<tr>
<td>ProCA1.G10</td>
<td>15.0</td>
<td>97.86</td>
<td>18.82</td>
</tr>
<tr>
<td>ProCA1</td>
<td>10</td>
<td>134</td>
<td>14.94</td>
</tr>
<tr>
<td>ProCA1.B10</td>
<td>6.00</td>
<td>122.8</td>
<td>9.45</td>
</tr>
</tbody>
</table>
SDS-PAGE analysis of each purified ProCA1 variants indicates that the proteins were isolated in its pure form (Figure 3.11 B). However, two smeared bands at 12 kDa for ProCA1.G10 suggest that there was degradation. This was further supported by the western blot result, as western blot is a sensitive technique that can identify a very tiny amount of protein with a series of primary and secondary antibodies bound to it (Figure 3.11 A). For ProCA1.B10, a bright band is observed at around 24 kDa, which can be justified as a dimer form. Although ProCA1.B10 does not contain cysteine ruling out the possibility of forming a disulfide bond, this dimer could have possibly formed due to protein-protein hydrophobic interactions.

3.1.3. Determination of Relaxivity

As discussed in chapter 1, relaxivity is a technical term used for MR Imaging to describe the ability of a contrast agent to utilize paramagnetic metal ions and increase the relaxation rate of the surrounding water proton spins per concentration of a contrast agent under magnetic field[58]. Many factors affect the relaxivity of a contrast agent such as the strength of magnetic field, interactions with water and gadolinium, molecular size, flexibility or rigidity of the contrast agent, protein-metal interactions. In this part of the work, relaxivity of ProCA1 variant was measured using 60 MHz relaxometer (Bruker). Each protein samples were chelaxed to eliminate free metal ions present in the solution that may cause hindrance. Each ProCA1 variant was incubated with various concentrations of Gd$^{3+}$, ranging from 1μM to 50 μM in 10 mM HEPES at pH 7.2. ProCA1 variants have one engineered binding pocket containing negatively charged residues that can interact with the Gd$^{3+}$ and water molecule. These interactions in the presence of a magnetic field are measured as T1 and T2 relaxation times, and the relaxometer gives these values in microseconds (μs). The T1 and T2 measurements of the buffer with no Gd$^{3+}$ were used as a control. The relaxation rate is inversely proportional to the relaxation time.
(1/T1 or 1/T2). These measurements were plotted on a graph as a function of the Gd^{3+} in molar concentration. The R1 and R2 relaxivities were calculated using Equation 3-1

\[ R_{1,2} = \frac{\frac{1}{T_{1,2 \text{Sample}}} - \frac{1}{T_{1,2 \text{Buffer}}}}{[\text{Gd}^{3+}]_{\text{Total}}} \]  

Equation 3-1

The relaxivity of ProCA1 variants measured at three different temperatures, 25°C, 30°C and 37°C are shown in Figure 3.12. Relaxivity of a contrast agent can be affected by a change in temperature. As the temperature increases, the tumbling rate of a contrast agent increases causing an increase in the relaxation times. All ProCA1 variants showed much higher relaxivities (25.9-49.2 mM^{-1}s^{-1}) as compared to the clinical contrast agent such as Magnevist (Gd-DTPA: 3.5 and 5 mM^{-1}s^{-1}). ProCA1G10, ProCA1B10 and ProCA1B14 have much higher relaxivities than that of ProCA1. Such drastic difference in the relaxivity could be due to the additional targeting sequence grafted onto the scaffold protein influencing the tumbling and movement of the contrast agent. In addition, it can also be concluded that the linker connecting the grafted peptide and the scaffold ProCA1 enables the ProCA1 moiety to move freely without being hindered or restricted by the grafted peptide on the C-terminal of ProCA1. This indicates that ProCA1 variants require less local concentration or dosage to improve the contrast of tissue compared to that of clinical MRI contrast agents. Due to this high relaxivity, our contrast agent can target the cancer biomarkers that have $10^3$-0$^6$ receptors per cell and give out detailed information with improved sensitivity of MR images and high resolution.
Figure 3.12 Relaxivity measurements of ProCA1 variants.

(A) The relaxivity of ProCA1 variants (ProCA1, ProCA1.G10 and ProCA1.B10) measured at 25°C. (B) The relaxivity measurements of PRoCA1 variants at 30 °C. It’s clear that the relaxation property of the targeted contrast agent is enhanced due to the additional sequence. (C) The relaxivity measurements of ProCA1 variants at 37 °C compared with the relaxivity of Magnevist (Gd-DTPA) a commercially available contrast agent. Relaxivity of ProCA1 variants are much high compared to Gd-DTPA. There is no significant difference in the relaxivity of ProCA1.B10 and ProCA1.B14 despite of the sequence differences. Non-targeted ProCA1 seems to have a decrease in the relaxivities, this could have been either due to an error in preparing samples or the quality of the protein has been affected due to degradation.
3.1.4. Fluo-Zn competition assay

Zinc is one of the major physiological metal ions, which is potentially involved in transmetallation and thereby possibly removing the Gd$^{3+}$ from contrast agents. The clinical MRI contrast agent DTPA has a relatively high affinity for Zn$^{2+}$ (6.3 × 10$^{-19}$ M), to that for Gd$^{3+}$ (1.9 × 10$^{-21}$ M) (NIST). To further understand and analyze any possibility of competition amongst the two ions in vivo, it is crucial to test the metal selectivity of our contrast agent. Dr. Yang’s Lab has previously studied binding affinities of ProCA1 with various metals, and the reported $K_d$ of ProCA1-Zn$^{2+}$ is 1.9 × 10$^{-7}$ M [29]. The Zn$^{2+}$ binding affinity of targeted ProCA1 variants were determined using Fluozin-1 competition assay as described in chapter 2 (section 2.11). Initially, one μM of zinc and one μM of Fluozin-1 dye were incubated in 50 mM HEPES, and 150 mM NaCl at pH 7.2 and their fluorescence spectra were obtained. Then, various concentrations of ProCA1 variants (ProCA1.B10 or ProCA1.G10) were titrated to the buffer-dye solution. The contrast agent competes with the dye for Zn$^{2+}$, causing a decrease in the fluorescence intensity (Figure 3.13). The binding affinity for zinc is calculated using the following Equation 3-2

\[ K_{d2} = K \times \frac{K_{d1}}{K_{d1} + [\text{Fluozin-1}]_T} \]  

\[ \text{Equation 3-2} \]

$K_{d2}$ is the apparent $K_d$ of contrast agent for Zn$^{2+}$, and $K_{d1}$ is the binding affinity of the dye Fluo-Zn1 for zinc and the total concentration of Fluozin-1. The zinc-binding affinity of ProCA1.G10 and ProCA1.B10 were thus determined to be 2.51 x 10$^{-6}$ M and 1.08 x 10$^{-6}$ M respectively.
Figure 3.13 Determination of Zn\(^{2+}\) binding affinity of ProCA1 variants using Fluo-Zn1 dye competition assay.

Fluorescence spectra of zinc binding competition assay for (A) ProCA1.B10 and (C) ProCA1.G10 using FluoZn-1 dye.
3.2. Conclusions

A rapid and efficient method to express and purify the first generation of ProCA and its targeted variants has been successfully established by isolating the protein from inclusion bodies. Suitable competent cells, appropriate temperature and dosage of inducer are important key factors that affect the growth of bacterial cells, quality and the yield of the recombinant target protein. The first step, overexpression of ProCA1 in inclusion bodies has been successfully performed following the procedures designed (Chapter 2). As the data show, all ProCA1 variants were expressed well in *E.coli* BL21 (DE3) competent cells as inclusion bodies with the addition of 1 mM IPTG and temperature reduction to 25°C post induction. Although Tuner cell strains are well known to generate high amounts of inclusion bodies, somehow the inclusion bodies have an accumulation of DNA/RNA and nucleic acids fragments, which made the isolation of pure protein difficult.

Purification of the highly expressed proteins in inclusion bodies was performed. Protocols included several washing steps with Triton 100X. Triton 100X is a mild detergent that can solubilize lipids and cell membrane fragments that co-exist with the target protein in the inclusion bodies after cell lysis. Washing with 2 % Triton 100X was done until the appearance of the pellet changed from pale brown to white indicating the removal of unwanted cellular components. All ProCA1 variants yielded clean white pellets after three washes with Triton. In fact, such treatment enhanced the brightness of the band intensity in SDS-PAGE. The rapid expression of large amounts of these proteins in the bacterial system has high chances of generating misfolded protein. Hence, in our approach to solubilize the misfolded protein from inclusion bodies using denaturant such as 8 M urea plays in our favor. Urea not only unfolds and solubilizes the target protein but also helped in removing other unwanted proteins and
impurities. Furthermore, proper refolding of the protein can be done to achieve high quality. Refolding process was done by gradually reducing urea concentrations in the buffer that was used for dialysis. The following step was to purify using FPLC to remove any remaining impurities and nucleic acids. Using HiTrap anion exchange Q column, the protein eluted, as the salt gradient was introduced and increased up to 40%; nucleic acids eluted out at 100% salt gradient. As summarized in Table 3.1, the final yield of all ProCA1 varied from 6 mg/L to 19 mg/L. The tagless purification protocol requires repeated washing of the inclusion bodies and centrifugation after each wash, where a lot of protein is washed out along with the impurities, as is apparent in the 15% SDS-PAGE gel patterns.

The GRPR-targeted ProCA1 variants have high R1 and R2 relaxivities at 25°C, 30°C and 37°C in comparison with the Gd-DTPA, a commercial contrast agent. In fact, the relaxivities of the targeted variants is much higher than that of non-targeted ProCA1. Such marked difference in the relaxivity could be due to the additional targeting sequence grafted onto the scaffold protein perhaps influencing the tumbling movement of the contrast agent. Such high relaxivities suggests that our designed contrast agent, ProCA1 might have higher sensitivity, and thus could achieve enhanced images at lower local concentrations. In addition, the zinc-binding affinity of the targeted ProCA1 variants are very similar to the reported $K_d$ for ProCA1- Zn$^{2+}$, in the range of $10^{-6} – 10^{-7}$, and much weaker compared to the reported Gd$^{3+}$ binding affinity of ProCA1 in range of $10^{-13}$ M[29]. The zinc binding affinities are preliminary and should be repeated in future along testing the gadolinium binding affinity of the targeted ProCA1.
4. OPTIMIZING THE EXPRESSION AND PURIFICATION OF HUMANIZED PROCA32 AND ITS MUTANT

4.1. Introduction

As discussed in chapter 1, ProCA32 is further humanized with engineered mutations on the human sequence of α parvalbumin. In order to obtain a high quality contrast agent for the biophysical characterization and animal model studies, it is extremely important to design a well-established, time-effective, cost efficient protocol for achieving high yields of purified humanized ProCA32 (hProCA32) agents. In this chapter, the expression results will reveal that the newly designed humanized contrast agent was successfully over expressed in Luria-Bertani broth medium, M9 minimal medium and various *E.coli* Cell strains using the aforementioned expression protocol in the Chapter 2 (Section 2.3). The hProCA32 was purified following Dr. M. Henzl’s protocol with modifications as mentioned in Dr. Shenghui Xue’s dissertation.[39, 59] This protocol, previously established for expression and purification of parvalbumin by Henzlet.al, demonstrates that hProCA32 can be effectively isolated and purified via tagless purification from nucleic acids coexisting in the supernatant following cell lysis. Studies were performed to improve the product yield during the purification process by overcoming the limitations associated with the separation of metal bound (holo) and metal free (apo) using EGTA in the buffers. Subsequently, experimental analysis of the expression of $^{15}$N isotope labeled hProCA32 was also performed to improve the expression levels using M9 minimal medium.
4.2. Results

4.2.1. Developing the Expression and Purification of hCA32 based on previously optimized protocol of rat ProCA32.

As discussed in section 2.3, the plasmid of hCA32 cloned into pET-22b (+) vector was first used for small-scale expression in bacterial cells to analyze the optimal conditions for overexpression of protein. A typical process for bacterial growth of E.coli cells has four stages: lag phase, log phase, stationary phase, and death phase. The cellular growth curves in Figure 4.1 indicate that the bacterial cells of both tested E.coli strains, Bl21 (DE3) and Tuner, grew fairly well in LB medium at 37 °C and at 25 °C after induction. Samples for SDS-PAGE analysis were collected at various time points, before and after induction of each expression system. SDS-PAGE gel analysis of Bl21 (DE3) cell strain indicates that there was little desired hProCA32 expressed prior to IPTG induction for all three selected colonies. As discussed earlier in Chapter 3, such protein expression in the absence of inducer is known as leaky expression. Similar results were observed for the Tuner cell strain for colonies 1 and 3. For colonies 1 and 3, bright bands around 12 kDa on SDS gel after induction suggests that the protein was indeed expressed however the yield seemed to be relatively low (Figure 4.1 B, right). All three colonies for BL21 (DE3) cell strain seemed to have consistent expression, whereas Tuner cells seemed to have quite a different growth amongst the three colonies (Figure 4.1)
Figure 4.1 Cellular growth curve for the small scale expression of hCA32 in (A) BL21(DE3) (B) Tuner and their corresponding 15 % SDS-PAGE.

A) Small scale expression of hProCA32 transformed in to BL21 (DE3) cell strain; three different colonies were picked and expressed in 10 mL volume of LB medium. Cellular growth curve of the OD absorbance values at various time points of the expression and 15 % SDS-PAGE analysis of the samples collected before and after induction. (B) Small scale expression gel of three colonies of hProCA32 in Tuner cell strain in 10 mL LB medium. Cellular growth plot at various time points during the expression and 15 % SDS-PAGE gel of the samples before and after induction.

*Note: All the samples added to gel electrophoresis are of equal volume of the culture.
Due to the consistency of the protein expressed after induction, BL21 (DE3) cell strain was further used for overexpression of hProCA32. BL21 (DE3) cell strain is a mutant, derived from BL21 that activates the T7 promoter site during the induction process[56]. In order to optimize the expression level of hProCA32 and eliminate the expression of unwanted proteins, another mutant of BL21, BL21 (DE3).pLysS was also used. BL21 (DE3).pLysS cell strain has a pLysS mutation that allows the release of the T7-Lysozyme, which reduces the expression of unwanted proteins prior to induction.

Following the successful transformation of pET-22b (+)-hProCA32 gene into BL21 (DE3) and BL21 (DE3).pLysS competent cells, the procedure implemented for the expression of the humanized contrast agent following the method described in the Section 2.3. By the increasing optical density of the cell culture, it can be concluded that the bacterial cells of both competent cells grew fairly well at 37 °C before induction and at 25 °C after induction (Figure 4.2A and C). This was further evident by the increasing cloudy appearance of the medium with time. For this particular batch of expression, flask 3 for the BL21 (DE3) cells seem to have no change in the OD absorbance prior to IPTG induction indicating that the bacterial growth was decreased due to possible contamination as same procedures were used for other the mediums in other flasks. 15 % SDS-PAGE gel in Figure 4.2 B and D shows faint bands around 12 kDa prior to induction in both competent cells indicative of leaky expression. The heavier band on the gels corresponding to samples 3 hours after IPTG addition indicates that a significant amount of hProCA32 protein was overexpressed. However, the density of the band was reduced in the case of the overnight induction at low temperature. In the high amount of bacterial cell pellets, lipids, DNA and other cellular components are expressed in large quantity along with the of desired protein. Due to the presence of lipids and DNA, the SDS sample prepared from
the cell pellets becomes sticky, making it difficult to solubilize in the SDS sample buffer even after boiling and reducing its resolution. This can provide an explanation for the faint bands in SDS gel for the overnight samples. However, the high optical densities values after the overnight induction suggest that indeed bacterial cells were overexpressed.

Figure 4.2 The Large scale expression of hCA32 in *E.coli*. (A) (B) Cellular growth curve and 15 % SDS PAGE hCA32 expressed in BL21(DE3) respectively and (C)(D) Cellular growth curve and 15% SDS PAGE of hCA32 in Bl21(DE3).pLysS.
Humanized contrast agent was further purified using Dr. M. Henzl’s protocol for wild-type alpha parvalbumin, with some modifications made by Dr. Xue Shenghui, and as describe in section 2.4. The high thermostability and water solubility of hProCA32 can be used to our advantage for purifying hProCA32 in its pure form. Recent research has shown that high negative surface charge has a strong correlation with an increased protein solubility.[60] Due to high negative charge, hProCA32 is highly soluble and practically stays in the supernatant after breaking the cell by sonication. On the other hand, nucleic acids are also highly negatively charged and remain soluble in the supernatant. The main goal is to isolate a well-folded, pure and functional form of this contrast agent. After breaking the cells in lysis buffer containing protease and benzonuclease, the mixture was centrifuged to collect the supernatant. Although hProCA32 is soluble, there are some other unwanted proteins that also soluble and remain in supernatant after sonication as seen in the SDS-PAGE gel (lane 2) Figure 4.4 where multiple protein bands are seen between 25 kDa to 68 kDa. Taking advantage of the thermostability of hProCA32, the supernatant was heated in boiling water at 80-90 °C for 20 minutes to precipitate out unwanted proteins. Figure 4.3A-B (lanes 4 and 5) demonstrates that the majority of the unwanted proteins were eliminated out in the pellet after heating it for 20 minutes leaving hCA32 in the supernatant. Experiments performed by previous lab members on rat ProCA32 showed the presence of high amount of DNA/RNA even after the addition of benzonuclease in the lysis buffer[39, 61]. So, the next step is to separate the substantial concentrations of nucleic acids that coexist along with the contrast agent (verified by UV absorbance). The supernatant was treated with 3% streptomycin sulfate (optimized concentration), which is an antibiotic that binds to the nucleic acids and precipitates them out. The supernatant was again heated for another 20 minutes to get rid of any possible unwanted proteins and dialyzed in 10 mM HEPES
pH 8.0 prior to purification by anion exchange on a Q-column.

Figure 4.3 The purification gel of hCA32 based on Dr. M. Henzl’s protocol modified by Dr. Shenghui Xue for rat ProCA32.

Samples taken throughout the purification of hProCA32 expressed in A) BL21 (DE3) and B) BL21 (DE3).pLysS competent cells were applied to 15% SDS-PAGE. Each lane are Labeled and are represented as follow: (CP) Pellet after sonication, (SP) Supernatant after sonication, inclusion body after heating for 20 minutes, supernatant after heating for 20 minutes, inclusion body after the addition of streptomycin, Supernatant after adding streptomycin, Cell pellet after heating it for 2\(^{nd}\) time, supernatant after heating for the 2\(^{nd}\) time, supernatant after dialysis twice overnight.
Since hProCA32 is originally a calcium binding protein, it may bind to free calcium ions that are naturally present in the purification buffers and the presence of a mixture of calcium bound form and calcium free form in the protein solution might affect the Gd\(^{3+}\) binding and skew the results of metal binding studies. The original FPLC purification protocol for removal of DNA/RNA from the protein solution included two rounds of FPLC, in the presence and absence of Ca\(^{2+}\) and EGTA, however, large amount of protein was lost during these sets of FPLC runs. In order to effectively remove large amounts of DNA/RNA and small nucleic acid fragments, previous lab member Dr. Shenghui Xue simplified the purification protocol for rat ProCA32: 1) optimizing the percent of streptomycin sulfate to precipitate DNA from the protein solution, 2) increasing the pH of the FPLC buffers from 7.5 to 8.0 for improving the binding of protein to the anion exchange column and 3) eliminating calcium and EGTA from the FPLC buffer. In order to test these conditions for hProCA32, the next step is to isolate the protein from remaining wanted cellular components and DNA/RNA fragments by anion exchange Q column using FPLC. Q column was cleaned and charged with buffer B (10mM HEPES, 1M NaCl pH8) followed by Buffer A (10 mM HEPES pH 8.0). At first 10 mL of dialyzed contrast agent was injected into the FPLC system. The protein was purified following the program in Figure 4.4, in which the injected protein first binds to the column. Subsequently, the unbound components were washed with 5-column volume (CV) of buffer A (10 mM HEPES pH 8.0). Slowly, buffer B (10 mM HEPES 1M NaCl pH 8.0) concentration was increased to 25 % within next 4 CV followed by a steady increase to 35 % NaCl, salt concentration for next 2 CV. Further, the NaCl concentration was held for 3 CV before a sharp increase to 100% NaCl concentration to elute any possible component, protein and nucleic acids bound to the Q column. Finally, Q- column was re-equilibrated by washing it with 100 % buffer A for 5 CV.
Figure 4.4 FPLC salt gradient program for hCA32 purification.

Figure 4.6A and Figure 4.7 depicts a typical chromatogram from Q- column FPLC separation of hCA32 expressed in BL21 (DE3) and BL21 (DE3).pLysS respectively. The UV absorbance at 280 nm from FPLC detector demonstrates four to five peaks as the buffer B (10mM HEPES, 1M NaCl pH8.0) elution increases. Fractions eluted under each peaks were collected and tested by UV- Vis spectroscopy and 15 % SDS PAGE. Contrast agent expressed in both competent cells, BL21 (DE3) and BL21 (DE3).pLysS seem to have similar chromatogram. Referring to the UV spectrum of peak 1 (Figure 4.6) with high absorbance at 260 nm and absence of band in SDS PAGE it can be concluded that peak 1 is more likely to consist of some nucleic acid fragments that were unable to bind to the column. As discussed earlier, the presence of calcium bound protein in the purified hProCA32 may affect its Gd\(^{3+}\) binding. Preliminary FPLC results for hProCA32 demonstrated that it not only separated DNA and nucleic acids fragment but holo and apo form of the contrast agents were also separated during the anion exchange chromatography using FPLC. Anion exchange column separates components based of the negative charge; hence a minor difference in the charge may elute the component under separate peaks. The presence of separate peaks on the FPLC chromatogram
suggests that the eluted protein is in different forms having varied characteristics. The holo-form (calcium bound) of the contrast agent will have less total negative charge compared to the apo-form (calcium free) of the contrast agent. Thereby, the calcium bound form elutes out first (Peak 2) when the salt gradient is first introduced into the program followed by the calcium free form (Peak 3) (Figure 4.6 A and 4.7). The apo form of the contrast agents can be readily isolated under separate peak that can be further used for its biophysical characterization and metal binding studies. The UV-Vis spectrum in Figure 4.6C shows high absorbance at 260 nm that are equally plateauing at 280 nm. It is evident that the desired protein is present but with equal concentrations of nucleic acid fragments. The purification protocol for rat ProCA32, previously established by Dr. Shenghui Xue suggests these nucleic acid fragments could be removed by concentrating the solution several times using amicon nitrogen concentrator with 3kDa membrane. Uv-vis spectrum in Figure 4.6 and Figure 4.7 clearly shows a high absorbance at 280 nm after concentration, indicating the elimination of nucleic acid fragments. The Uv spectra obtained are closely related to the published spectrum of rat alpha parvalbumin as shown in the Figure 4.5 with calcium free and calcium- loaded form.[62] For BL21 (DE3), Peak 2, 3 and 4 shows a high-density band in SDS PAGE gel and a signature UV spectrum with evident shoulder at 285-295 nm. The Uv spectrum of peak 2 has a spiky shoulder at 285-295 nm indicative of the presence of holo, calcium bound form of the contrast agent and peak 4 with a flattened shoulder at 285-295 nm consists of apo, calcium free form of the contrast agent. Since peak 3 is not separated well, it may contain a mixture of holo and apo form. Even though the FPLC elutes out the contrast agents under separate peaks, the final yield of the apo form of the contrast agent is affected as only the apo form can be used for metal binding and animal
experiment. So, further optimization of the FPLC purification process is necessary to obtain a pure form of hProCA32.

Figure 4.5 The Uv spectrum of calcium free (dashed line) and calcium loaded (solid line) form of Parvalbumin F102W

However, in case of protein expressed in BL21 (DE3),pLysS it is evident from the FPLC chromatogram that both peaks 2 and 3 are separated well. The SDS PAGE gel confirmed the presence of protein in both peak 2 and 3. The distinct UV spectrum of Peak 2 with spiky shoulder at 285-295 nm more likely corresponds to the holo form and peak 3 with flattened shoulder indicates the presence of apo form of the contrast agent. The yield obtained for humanized ProCA32 by using the protocol established for rat ProCA32 are relatively low compared to the one for rat ProCA32 (40-60 mg/L). The contrast agent expressed in BL21 (DE3) had final yield of about 19.25 mg/L (apo form) and for BL21 (DE3). pLysS was 13.12 mg/L (apo form). In order to overcome this issue and increase the final yield of the metal free form of the contrast agent, the purification protocol was further modified by two approaches: 1) addition of EGTA in the purification buffers. 2) By elongating the salt gradient and holding for a longer time when the peak 2 and 3 are eluting.
Figure 4.6 A typical FPLC chromatogram of hProCA32 by anion exchange Q-column separation, the 15 % SDS-PAGE and UV absorbance of each peak eluted for hProCA32 expressed in BL21 (DE3) competent cells.
Figure 4.7 A typical FPLC chromatogram of hProCA32 by anion exchange Q-column separation, the 15% SDS-PAGE and UV absorbance of each peak- hProCA32 expressed in BL21(DE3).pLysS
Another batch of hProCA32 was expressed in BL21 (DE3) and BL21 (DE3) competent cells in LB medium following the aforementioned procedure in section 2.3. The results for hProCA32 expressed in BL21 (DE3) are used for the illustration. The optical density of the medium was measured at several time points, and the cell culture was induced when the optical density reached 0.6 to 0.8. The temperature was reduced from 37°C to 25°C once the cell culture was induced. Samples were collected before IPTG induction, and after 2 hours, 5 hours and overnight induction for SDS-PAGE analysis (Figure 4.8A). Bright protein band at around 12 kDa on 15 % SDS-PAGE gel indicates the expression of hProCA32. It can be concluded that the optimal condition for the expression of hProCA32 is harvesting the cell pellet 5 hours after IPTG induction. The protein remained in the supernatant throughout the purification process once the cells were broken by sonication as confirmed by the SDS-PAGE (Figure 4.8 B). The purification procedures are same until the second time heating of the supernatant. hProCA32 was dialyzed in 10 mM HEPES, 5mM EGTA at pH 8.0 for 24 hours to entirely remove streptomycin sulfate before performing anion exchange chromatography. The buffer was changed after 1 hour, and again after 4 hours, and then allowed to equilibrate overnight. The next day, the protein solution was collected and centrifuged to remove possible precipitates. The protein solution was further incubated with 10 mM of EGTA for 24 hours prior to FPLC. As seen in the FPLC chromatogram, the majority of the protein indeed elutes out under peak 3 (Figure 4.9A) and further supported by a bright and thick band at 12 kDa on 15 % SDS-PAGE gel (Figure 4.9 B). The fractions eluted in peak 2, and 3 were concentrated using an amicon nitrogen concentrator with 3kDa membrane. The Uv spectrum of peak 2 and 3 after concentration showed high absorbance at 280 nm and low absorbance at 260 nm indicating the removal of nucleic acid (Figure4.10).
Figure 4.8 15 % SDS-PAGE gel of hProCA32 (A) expressed in BL21 (DE3) and (B) purification.

(A) SDS-PAGE gel of samples before and 2 hours, 5 hours and overnight after induction for the expression of hProCA32. (B) 15 % SDS-PAGE gel of the samples collected during each step of purification. The protein remained in the supernatant after sonication. Dialysis buffer prior to FPLC contained 10 mM HEPES, 5 mM EGTA at pH 8.0

Figure 4.9 A typical FPLC chromatogram of hProCA32 purified by anion exchange chromatography and the 15 % SDS-PAGE of the fraction corresponding to the chromatogram.

(A) FPLC chromatogram of hProCA32 incubated with 10 mM EGTA prior to the injection into FPLC. Majority of the protein elutes out under Peak 3. (B) 15 % SDS-PAGE gel of fractions under each peak
It is clear from the UV spectrums that proteins eluted from both the peaks are in apo-form due to the presence of flattened shoulder at 285-295 nm. (Figure 4.10 A) In order to confirm this, 5 mM of calcium solution was added to the protein solution from each peak and UV spectrum was obtained. As expected, after the addition of calcium to the protein solution the absorbance of the shoulder at 285-295 nm spiked up, indicating the presence of calcium bound protein (Figure 4.10 B and C). A similar phenomenon was observed after the addition of 5 mM gadolinium to the protein solution suggesting its binding to the protein (Figure 4.10 D). Incubating the contrast agent with 10 mM EGTA for 24 hours prior to anion exchange chromatography indeed played a significant role in improving the yield of calcium free form of the contrast agent. The calcium-free form of the contrast agent eluted as peak 3 can be used for biophysical characterization and animal experiments. The final yield of the proteins eluted out under peaks 3 was 64.2 mg/2L. The final total yield of the calcium-free form of hProCA32, using the modified protocol was 32.1 mg/L.
Figure 4.10 UV Spectrum of the protein purified by anion exchange chromatography.

(A) The UV spectrum of peak 2 (red) and 3 (black) after concentration, the low absorbance of the shoulder from 285-295 nm indicates that the protein eluted under both peaks is in apo form, calcium free form. (B) UV spectrum of peak 2: calcium free form (black) and calcium loaded form (red) with addition of 5 mM calcium. (C) UV spectrum of peak 3: calcium free form (black) and calcium loaded form (red) with addition of 5 mM calcium. (D) UV spectrum of peak 2: calcium free form (black), Gd$^{3+}$ loaded form (red) with addition of 5 mM gadolinium, metal loaded form (green) with addition of 5 mM gadolinium and 5 mM calcium.
4.2.2. Optimizing the expression and purification for $^{15}$N labeled hCA32

Nuclear magnetic resonance (NMR) spectroscopy is a valuable and reliable tool to study the structure and function of biological molecules. Detailed analysis of structure and dynamic properties and interaction of various compounds and biological molecules with small molecular weight has become possible with NMR. NMR can even detect minute changes in the structural characteristic of a biological molecule, such as ligand binding, intra-molecular interactions and many more, by changes in chemical shift. Thus, NMR can be helpful in order to support the data obtained by fluorescence spectroscopy for determining the dissociation constant of our contrast agents to different metal ions like Ca$^{2+}$, Zn$^{2+}$ and Tb$^{3+}$ etc. by monitoring various chemical shifts. The relaxivity of our contrast agent is 10 times higher than that of the commercially available contrast agents, and it can possibly be used for the multimodal molecular imaging techniques. Here, NMR can be of great help to determine the dissociation constant of our designed contrast agent to other lanthanides such as Lu$^{3+}$ and Ga$^{3+}$ that are widely used for other forms of imaging modalities. Isotope labeling of organic and biological molecules has been often used to empower and further enhance the detection ability of NMR, as the introduction of isotopic nuclei can eliminate the coupling effects, which reduced the complexity of the spectrum. Uniform $^{15}$N labeling of biological molecules is widely used, as the amount of nitrogen needed for the cellular growth can be readily controlled making the sample preparation task easier and increases the resolution of the NMR spectra. [63]Thus, the changes in the chemical shift of the isotope labeled hProCA32 can be monitored to attain its binding affinity for metal ions. The primary objective of this part of work is to optimize the expression condition of the $^{15}$N-labeled hProCA32 and attain higher yield and higher quality protein for NMR experiments.
First, the contrast agent is expressed in M9 minimal medium with non-labeled NH₄Cl for preliminary experiments to investigate its growth in M9 medium, which has fewer nutrients compared to LB medium. Since, the labeled isotopes are relatively costly; it is best to test the optimal conditions in the non-labeled medium. The M9 minimal medium contains Na₂HPO₄, KH₂PO₄, FeSO₄, MgSO₄, CaCl₂, micronutrients, NaCl, thiamine, and vitamin supplements (Detailed ingredients of M9 minimal medium are described in the Section 2.3.1.) The contrast agent was expressed in M9 minimal and in LB medium using BL21 (DE3).pLysSE.coli cells. The protein expressed well in both medium (Figure 4.11 B) and further confirmed by the western blot (Figure 4.11 A). The protein expressed in M9 was purified to attain the final yield of 11.09 mg/L (22.18 mg/2L), which is comparable with the protein expressed in LB medium.
Figure 4.11 Overexpression and purification of hCA32 in M9 minimal medium (A) Western blot (B) 15 % SDS PAGE of hCA32 expressed in M9 minimal medium and LB medium. (C) Purification of hCA32 from M9 medium (D) UV spectra of pure hCA32.

Next step is to express the contrast agent using $^{15}$N-labeled medium and to isolate high quality, well-folded hProCA32 for NMR studies. The contrast agent was expressed in M9 minimal medium, as well as in LB medium as a control, following the protocol described in the Methods and Materials section. The cellular growth curve in M9 minimal medium shows much slower growth rate compared to the bacterial growth in LB medium presumably due to the lack of nutrients in M9 medium (Figure 4.12 A) The protein was expressed at 25 °C for 6 hours after induction, once the optical density reached 0.6. The SDS-PAGE shows the expression of the protein with time: bright bands at 12 kDa gets heavier 2 hours after induction and 6 hours
after induction. This indicates that reducing the temperature after inducing the cells (before induction 37 °C to after induction 25 °C) helped in proper folding and significantly enhanced the overexpression of the protein. This was further confirmed by western blot. Two distinct bands at 12 kDa are indeed protein bands as the antibodies used in western blot are specific to hProCA32. As expected from the previous purification results, protein remained soluble throughout the purification process and the unwanted proteins were precipitated during the heating of protein solution (Figure 4.13). SDS-PAGE of the eluted fraction from anion exchange Q column indicates that the majority of the protein remained in Peak 2 and 3 (Figure 4.14B). This was supported by western blot analysis; however, smaller amounts of protein also eluted out in peak 1(Figure 4.14 C) as bands at 12 kDa are observed in western blot. As mentioned earlier, western blot is a highly sensitive technique that employs antibodies specific to the biomolecule of interest and can detect tiny amounts of the target molecule, however, it is not as quantitative as compared to Coomassie blue staining. Coomassie blue stained-SDS PAGE gel can detect as low as 50 ng of protein and since no protein bands for peak 1 are visible on the SDS gel such minute loss can be ignored. Peak 2 and 3 were concentrated to remove any possible presence of nucleic acids fragments in the solution. NMR is highly sensitive to salts; presence tiny amounts of salts can compromise the resolution of the spectrum. The concentrated protein samples were passed through a desalting column used to get rid of salts. The UV spectra obtained are closely related to a typical hProCA32 spectrum, a flattened shoulder at 285-290 nm indicates the absence of free metal ions. Although, high amount of protein was expressed, a lot of it was lost during the purification process reducing the final yield of the protein. Either due to the repeated centrifugation process, inefficiency of the contrast agent to bind to the Q- column
due to insufficient cleaning of desalting column due to human error. The final yield of the $^{15}$N labeled contrast agent was calculated to be 10.97 mg per 2 liters of expression medium.

Figure 4.12 Over expression of $^{15}$N labeled hCA32 using M9 minimal medium.

(A) Growth curve of bacterial density with time dependence: Flask1 and 2: $^{15}$N labeled M9 medium, Flask 3: LB medium (B) 15 % SDS-PAGE of $^{15}$N labeled hCA32. (C) The western blot corresponding to the SDS-PAGE gel in (B). (D) The 15% SDS-PAGE of the protein expressed in LB medium.
Figure 4.13 The purification of $^{15}$N labeled hCA32 expressed in M9 minimal medium.

Figure 4.14 The purification of $^{15}$N labeled hCA32 using FPLC. (A) FPLC chromatogram (B) 15 % SDS PAGE (C) Western Blot (D) UV spectra of the fractions eluted by FPLC Q column.
4.2.3. Molecular cloning of hCA32.cys

Development of therapeutic protein drugs is the new emerging field that has been revolutionized in the past few decades[48]. The recombinant technology and protein engineering have been used to alter and enhance the native properties of a desired protein and utilize it for the disease related treatments. For instance, recombinant human insulin is used to treat diabetes mellitus, recombinant erythropoietin is used to treat anemia due chronic renal failure or chemotherapy and many more with special targeting and diagnostic properties. [49, 64] However, these proteins are not naturally evolved for therapeutic drug application, modifying them leads to an immune response by potential antibodies formations. Such immune response is referred to as immunogenicity. According to the guideline by FDA, there are several patient-specific factors and product-specific factors that influence immunogenicity that influence immunogenicity[65]. The patient-specific factors such as genetic background of the patient such as patient’s immunologic status, competence, sensitivity, tolerance, route, dose and frequency of the administration differ from patient to patient and are difficult to be studied. The product specific factors include: the origin of the protein (human or non-human sequence), stability, solubility (aggregation), and primary structure of the protein molecule,
glycosylation, and PEGylation. The product related factors could be optimized and monitored while developing the protein drug. Humanization of a therapeutic protein drug is an important method to reduce the immunogenicity. Among our previously established ProCAs, ProCA32 seems to be the best candidate to be carried on to the next step of designing a therapeutic protein drug, and test biophysical properties. In our approach of testing the humanized ProCA32, PEGylation is another technique that is FDA-approved to reduce the immunogenicity effects. As mentioned earlier in chapter 1, when protein drug is conjugated with PEG, flexible and inert PEG chains wraps up the antigenic epitopes of the drug molecule reducing the toxicity and immunogenicity generated by foreign protein drugs and decreases the degradation caused by proteolytic enzymes without interfering in its therapeutic properties. [50] PEG is a hydrophilic molecule, which increases the solubility and increase in molecule weight help extend the half-life of the protein that enhances the stability with reduced renal filtration. Our previously PEGylated versions of ProCAs demonstrated an increase in the relaxivity due to increase in the molecular weight, changes in water properties and water-metal exchange rate due to hydrophilic PEG. Protein modification by PEG can be done on various residues, but the most frequently employed approach is PEG conjugation with free amine groups typically on lysine. However, the majority of proteins sequence consists of multiple lysine residues (hProCA32 consists of 16 lysine residues as seen in Figure 4.16) and PEG specific to amine can react with any free amine available in the sequence. This leads to the formation of multiple isomers of PEGylated-proteins with multiple amine bearing sites being modified. Separation of such heterogeneous mixture of PEGylated protein becomes difficult. If the modification of these therapeutic proteins does not attain reproducibility, the regulatory FDA approval becomes complicated. In order to overcome heterogeneous modification of our contrast agents, we followed another modification approach
by site-specific PEG conjugation at cysteine. This approach allows selective protein modification at a single or predetermined site with the homogenous outcome. Cysteine residues are mainly associated with disulfide bridges and formation of secondary structures; hence, accessible cysteine residues without any function are rarely available. A protein sequence containing free cysteine residue, which is not involved in the disulfide bond formation and does not play an important role in the protein functioning and activity can be used for modification. Moreover, a cysteine residue can be introduced in the protein sequence either replacing a non-essential amino acids from the protein sequence or insertion at a desirable position can be easily attained by site-directed mutagenesis. In case of hProCA32, a cysteine residue was inserted at the N-terminus to allow site-specific conjugation of a single PEG at thiol group on cysteine using maleimide-PEG. Forward and reverse primers were designed, as shown in the Figure 4.17A; DNA gel electrophoresis results in (Figure 4.17 B) shows a band at 6000bp corresponding to expected template DNA. This indicates that the molecular cloning experiment was successful. The plasmid construct was further confirmed by getting it commercially sequenced from GENWIZ. The DNA sequence obtained from the sequencing was analyzed and aligned by Blast and Clustalw. The DNA and protein sequence alignment in Figure 4.18 shows the desired mutation at C-terminal of hProCA32 was successfully inserted into the plasmid.
Figure 4.16 Possible Lysine (Cyan) and Cysteine (Beige) PEGylation sites on hProCA32.

Xray crystalized structure obtained from pdb file 1RK9 and modified using USCF Chimera S56D, D101E and F103W
Figure 4.17 Molecular cloning of hCA32.cys by PCR (A) Forward and Reverse Primer design for site directed mutagenesis of hCA32 (B) DNA gel electrophoresis of the plasmid obtained after PCR

Forward Primer: 5’CAC CCT GGT AGC AGA GTC CTG CTA ACT CGA CCA CCA C-3’
Reverse Primer: 5’GTG GTG GTG CTC GAG TTA GCA GGA CTC TGC TAC CAG GGT G
Expression and purification of hProCA32.cys

The next step is to express and purify the newly cloned hProCA32.cys, and to study its biophysical properties after pegylation. The mutated contrast agent hProCA32.cys was purified using the aforementioned protocol described in section 2.12.2. The apparent increase in the optical density of the cell culture suggests that the bacterial cells containing the genetic
encoding of hCA32.cys grew fairly well. A faint band at 12 kDa in 15 % SDS-PAGE gel depicts some amount of contract agent was expressed prior to IPTG induction indicating the occurrence of leaky expression (Figure 4. 19B). hProCA32.cys includes of a cysteine at its C-terminal which introduces the capability to form a disulfide bond thus forming a dimer. Surprisingly, most of the protein remained as a monomer. The OD absorbance plot and bright band at 12 kDa in SDS-PAGE indicates that a high amount of protein has been expressed after induction at 25 °C temperature.

The protein was isolated using the protocol implemented for the purification of hProCA32 as described in section 2.4. Similar to hProCA32, the mutated variant hProCA32.cys is expected to have high thermostability and solubility. After harvesting, the bacterial pellet was solubilized in 10 mM HEPES at pH 7.3 containing 1 μL benconuclease, PMSF and 5 mM DTT (Dithiothreitol, a reducing agent to keep the SH-group in reduced state). As expected, the protein remained in the supernatant after the bacterial cells were broken by sonication. As seen in Figure 4.20, most of the unwanted proteins vanished when the sonicated supernatant was boiled at 80-90 °C for about 20 minutes. Streptomycin precipitated out most of the coexisting nucleic acid. In order to analyze the in solution composition of the protein, SDS- PAGE samples were kept in non-reducing condition. After heating the supernatant second time some of the protein was visible as dimer, which is clearly observed in the purification gel (Figure 4.20B) at about 24 kDa. The band intensity in SDS-PAGE 12 kDa was enhanced by the dialysis of the contrast agent in 10 mM HEPES and 5 mM DTT at pH 8.0, most of the protein remained in its monomer form throughout purification due to the presence of DTT in the buffers.
Figure 4.19 Overexpression of hCA32.cys in *E.coli* (A) Cellular growth curve (B) 15% SDS-PAGE of hCA32.cys expressed in BL21 (DE3).pLysS and LB medium.

The anion exchange chromatography eluted five different peaks, and the SDS gel electrophoresis clearly indicated that most of the protein was present in either peak 2 or 3.
The high UV absorbance (peak1) in the binding step of FPLC clearly indicates that the desired protein is not binding to the column and eluting out prior to the introduction of the salt gradient in the FPLC program. This can be further confirmed by 15 % SDS-PAGE gel of the fraction eluted under peak 1, where a clear band is observed at 12 kDa. This could be due to many possible reasons but most commonly due to overloading of the sample as the presence of significant amount of protein in the solution may exceed the column capacity. High salt concentration present along with the contrast agent prior to the FPLC injection can reduce the binding efficiency of the column and subsequently reduces its capacity. Thus, some of the protein remained unbound to the column and eluted out first in peak 1. In order to check this possibility, the eluted fractions were diluted with water and re-chromatographed. The re-chromatographed spectrum looked similar to the first run except with low UV absorbance (Figure 4.21B), suggesting that the presence of high salt in the protein solution indeed hindered the column binding process.

The presence of three separate peaks suggests that the eluted protein with different forms may have varied characteristics. Inferring the UV spectrums of each peak, it can be implemented that the majority of the protein eluted out in peak 2 and 3. The UV spectrum of the fractions eluted under all of the five peaks has absorbance at 260 nm that equally plateaus at 280 nm. UV spectrum in Figure 4.22B clearly shows a high absorbance at 280 indicating the presence of protein.
Figure 4.21 (A) Typical FPCL chromatogram of hCA32.cys purified by Q- column separation, (B) The spectrum of re-chromatography of peak 1 from A(C) the UV absorbance and (D) 15 % SDS-APGE gel of each fraction.
Figure 4.22 UV absorbance of concentrated hCA32.cys

The structural characterization of purified hCA32.cys by UV spectroscopy demonstrated a sharp peak at about 285-290 nm that is consistent with the hCA32 purification results further confirming the presence of high quality protein. Although a relatively high peak at 260 nm is suggesting possible presence of nucleic acid, it can be removed by concentrating the protein using a 3kDa membrane. Fractions eluted under peak 2 and Peak 3 from FPLC were concentrated and according to UV absorbance and $\varepsilon_{280} = 7200 \text{ M}^{-1}\text{cm}^{-1}$, the total yield was 26.64 mg/L.

4.2.5. Purification of rat ProCa32 expressed using fermenter

A higher dosage of protein is required to study the imaging properties of ProCAs in large animals and humans as compared to mice. The large-scale production of our designed ProCAs is another stumbling block for commercialization. Although ProCA32 is a desired candidate, it must have reproducible quality and high efficiency during industrial production. The expression and purification process must be readily scalable with reduced production cost. For this purpose, 12 liters of ProCA32 was expressed in a bioreactor with well-established pH,
temperature, airflow and oxygen levels (Protocol in Dr. Shengui Xue’s dissertation) [39]. The cell pellet obtained was purified to ensure the protein quality and its relaxation properties.

The cell pellet was homogenized in the lysis buffer and sonicated to break the bacterial cells. ProCA32 is highly soluble hence after breaking the cells by sonication, and the initial step is to separate the protein from insoluble cellular components by centrifugation. The 15 % SDS – PAGE gel (Figure 4.23) shows that most of the insoluble cellular components were removed in the pellet and ProCA32 remained soluble in the supernatant. ProCA32 has a unique property of maintaining its stable conformation when exposed to higher temperature, thermo stable. Hence, heating the solution would be an efficient and suitable way for industrial production to remove the unwanted proteins that are not stable at high temperature. The solution was heated at 90°C for 20 minutes and centrifuged at 7000 rpm to precipitate out the majority of non-heat stable proteins in the pellet. As discussed earlier, the proceeding step of purification is to precipitate out nucleic acid by treating the supernatant with, streptomycin sulfate. Heating the supernatant at 90 °C for second time assured the removal of any remaining unwanted proteins. The protein was then isolated from remaining nucleic acid fragments and impurities by FPLC- anion exchange column with a salt gradient program. The nucleic acids have higher negative charge compared to protein; hence nucleic acid remains bound to the column strongly. As the percent salt concentration increases the protein that is bound the Q- column is competed by the salts present in the buffer and elutes out first. The FPLC chromatogram demonstrates that the injected protein solution eluted out in 5 different peaks (Figure 4.20). Each fragment was analyzed by 15 % SDS-PAGE as shown in the Figure 4.25. As the salt concentration (Buffer B-10 mM HEPES, 1M NaCl pH 8.0) increased to 25 % the protein eluted under Peak 2 and 3. The first step is the binding of the protein to the column in the presence of no salt buffer A (10 mM
HEPES pH8.0). Slight increase in UV absorbance at low salt concentration of FPLC run suggests that some of the protein remained unbound to the column and eluted out under peak 1, which was further confirmed by the presence of a band around 12 kDa revealed in 15 % SDS-PAGE and by UV-Vis spectroscopy. This may have occurred due to the presence of high concentration of nucleic acids along with the contrast agent reducing the binding efficiency of the column.

![Figure 4.23](image_url) The purification gel of ProCA32 based on the purification method from Shenghui Xue’s dissertation.

The 15 % SDS-PAGE gel shows all the samples taken throughout the purification process of the bacterial expression of the protein at 12 L scale.
Figure 4.24 FPLC Spectrum of anion exchange Q- column.

A clear and bright band at 12 kDa in 15 % SDS-PAGE gel indicates successful isolation of ProCA32 in fractions under the highlighted red and green box. The two distinct peaks 2 and 3 represents holo and apo form of the ProCA32, respectively.
Figure 4.25 UV-Vis Spectra indicates a successful separation of ProCA32 in 10 mM HEPES pH 7.2.

The fractions eluted under each peak were analyzed by 15 % SDS-PAGE and further confirmed by the UV-Vis spectroscopy. The gel indicates the presence of Protein in Peak 2 and 3. The elution under peak 1 shows a light band at 12 kDa suggesting that some of the protein remained unbound to the column. The UV-Vis spectrum of peaks 1, 2 and 3 were identical with a signature shoulder at 285-295 nm that is unique to ProCA32. The presence of this shoulder
4.3. Conclusions

Development of a protein based contrast MRI contrast agent is a challenging field. In order to design a protein based contrast agent, selection of scaffold protein prior to building a Gd$^{3+}$ binding pocket is extremely important. For the third generation of ProCA, we have selected parvalbumin (PV) as a scaffold protein, which is highly stable, soluble, and tolerant to mutation. Moreover, PV is naturally a calcium binding protein and hence is capable of binding lanthanides. PV has no reported interactions with other proteins or peptides. Previously, a series of the third generation of ProCA (ProCA3) were designed (by Dr. Shenghui Xue), out of which ProCA32 variant showed a significantly high relaxivity both in vitro and in vivo. In developing a protein based contrast agent next step would be to humanize it with the goal of reducing the immunogenicity effects in humans. We have designed ProCA32 using the human sequence of PV with necessary mutations.

In this chapter, the expression of humanized ProCA32 (hCA32) has been optimized using various *E. coli* cell strains. The protein seems to express well in BL21(DE3).pLyS and BL21(DE3) competent cells. Further, the protein was isolated from unwanted protein and nucleic acids by heating it twice. Along with the contrast agent, nucleic acids are highly stable at high temperature as well; streptomycin sulfate was used to precipitate nucleic acid from the solution. Although the majority of nucleic acids were removed, the remaining nucleic acid fragments were further removed by anion exchange chromatography. Since hProCA32 is originally a calcium binding protein, it may bind to free calcium ions that are naturally present in the purification buffers. The anion exchange column separates components based on the negative charge; hence a minor difference in the charge may elute the component under different peaks. The holo form (calcium bound form) of the contrast agent with less total negative elutes out first (Peak 2) when
the salt gradient is first introduced into the program followed by the apo form (calcium free form) Peak 3 which was confirmed by the UV spectrums. High quality apo form of the contrast agent was isolated using both competent cell types, but with relatively low yield (13-19 mg/L) compared to rat ProCA32 (50-70 mg/L). In order to improve the yield of the apo form of the protein the purification process was optimized with the addition of 5mM EGTA in the dialysis buffer and incubating the protein with 10 mM EGTA for 24 hours prior to the anion exchange chromatography. The majority of the apo form of the protein was extracted from peak 3, with the final yield of 32.1 mg/L.

The expression and purification of $^{15}$N labeled contrast agent were also optimized where the contrast agent was expressed in M9 minimal medium. Large amounts of protein were expressed; however a lot was lost during the purification process. The purification process must be further optimized to attain high yield. In our approach to reduce the immunogenicity effects, one of the techniques applied is a conjugation of the contrast agent with PEG. To obtain homogeneous and site-specific PEGylation, a cysteine residue was inserted at the C-terminal of our hProCA32. The purification of the mutated variant, hProCA32.cys was also optimized under reduced conditions with a final yield of 26 mg/L. The cysteine PEGylation of hProCA32.cys will be shown in chapter 5. Furthermore, the purification of rat ProCA32 expressed using large-scale expression system; fermentation yielded 4 mM of 4 ml of pure proteins per 5 g of the wet pellet.

In summary, the humanized ProCA32 and its mutant has been successfully optimized to over express and purify under the conditions mentions earlier (Chapter 2) with high yield. The purified contrast agent, hProCA32 is further used for characterizing it structural and biophysical properties in the following chapter.
5. RELAXIVITY, METAL BINDING, SELECTIVITY AND STABILITY OF ENGINEERED HPROCA32

5.1. Introduction

The primary focus of this chapter is to characterize the biophysical properties of the newly designed hProCA32 such as metal binding and selectivity, relaxivity and PEGylation. The criteria for an ideal contrast agent are high relaxivity, \textit{in vivo} stability and high gadolinium binding affinity, as well as selectivity for Gd$^{3+}$ over other physiological metals. If the binding affinity of the contrast agent for gadolinium is not strong enough, the metal can be released from the binding pocket, generating toxic effects which are caused by the accumulations of free gadolinium leading to nephrogenic systemic fibrosis (NSF) or nephrogenic systemic dermopathy (NSD) [28]. Since gadolinium is a trivalent lanthanide that has similar ionic radius to divalent calcium, it can easily bind to calcium binding pockets on protein molecules as Gd$^{3+}$ and Ca$^{2+}$ have similar coordination with a stronger preference for oxygen as a ligand. Binding of calcium to the contrast agent may hinder the imaging process \textit{in vivo} and on the contrary, if free gadolinium binds to other physiological calcium binding proteins, it can disrupt the dynamic biological functioning which required calcium [66]. Theoretically, the hProCA32 should have similar relaxivity to rat ProCA32 and higher compared to the commercially available small molecule contrast agents. Another important characteristic of a contrast agent is having an intact structure and stability against proteolytic cleavage by proteases under physiological conditions. Furthermore, to reduce the immunogenicity of the protein based contrast agent, the hProCA32 has been modified with a long polymer chain of PEG by conjugation.

The main objective of this chapter is to investigate the binding of physiological metals such as Ca$^{2+}$ and Zn$^{2+}$ and lanthanides such as Tb$^{3+}$ and Gd$^{3+}$ to humanized ProCA32. Relaxivity
measurements and serum stability of hCA32 are performed to further test its suitability as a contrast agent. Various lengths of PEG are conjugated to test its effect on physical characteristics of hProCA32.

5.2. Results

5.2.1. Site specific Lysine and Cysteine PEGylation

Addition of polyethylene glycol chains with various lengths (also known as PEGylation) has been frequently used in development of therapeutic protein drugs as a method to decrease the immunogenicity and renal excretion, improve the drug delivery, in vivo solubility, stability and biocompatibility [67]. Protein conjugated with PEG improves the biological functioning as it can increase the size of the protein-drug, which in turn reduces renal clearance (filtration by kidney) and subsequent increase in the circulation half-life and eventually, prolonging the blood retention and improving the drug delivery [50, 68]. Long hydrophilic chains of PEG (CH) also envelops the drug-protein and shields it from the antigenic epitope and helps in reducing the immunogenic response generated by the introduction of foreign biomolecules and improves the solubility. However, if the length of PEG is not optimized, it may mask the functioning site and affect the therapeutic and biological functioning of the protein drug. As discussed earlier in Chapter 4, our lab has performed PEGylation on first generation of ProCAs which demonstrated significant improvement in in vivo MR imaging and $r_1$ and $r_2$ relaxivities along with improved biocompatibility properties including dose efficiency, solubility and blood retention and decreased immunogenicity. [38] Up to date, lysine PEGylated of ProCAs has been used for the animal studies; however, lysine PEGylation generates heterogeneous solution due to the presence of multiple lysine residues in our contrast agents. In order to study and optimize the specific PEGylating sites and assess the homogeneity and its effects on the relative metal binding sites,
we have designed a mutant containing cysteine at the C-terminal of the protein. The molecular cloning, overexpression and purification of this mutant (hProCA32.cys) are described in chapter 4. In this section, the PEGylation of hProCA32.cys with various lengths of PEG reagents has been optimized to achieve the highest quantity of PEGylated reagent.

Following purification, 500 µL of protein was mixed with 1 to 10 volumetric ratio of TCEP in the presence of nitrogen gas. The mixture was allowed to react for 20 minutes followed by removal of TCEP by concentrating the protein using a 3kD filter membrane (Amicon). Meimide-PEG reagent was added with 1:5 molar ratios, and then the PEGylated protein was separated from free PEG reagent by concentrator. The protein was PEGylated with 0.710 kDa, 1.24 kDa, 2 kDa and 10 kDa PEG chains. PEGylated contrast agents, stained (2kDa and 10 kDa) by barium-iodine staining followed by SDS-PAGE gel, confirmed that the protein has been successfully PEGylated (Figure 5.1). Each PEGylated protein was further analyzed by the MALDI mass spectroscopy (Figure 5.2). An apparent difference can be observed in the molecular mass of protein before (Figure 5.2 A) and after PEGylation (Figure 5.2 B, D and E). The MALDI spectrum of hProCA32.cys conjugated with 2 kDa PEG.cys reagent has a broad range of peaks centered at 14.41 kDa due to the polydispersivity of PEG reagent.[67] Each polyethylene glycol (O-CH₂ –CH₂) unit has a molecular weight of 44.05 Da (g/mol). The difference between each peak in MALDI spectrum is about 44-45 Da corresponding to the different lengths of PEG. Such difference indicates that the PEG reagent consists of a heterogeneous mixture of various lengths of PEG units. The methionine group in hProCA32 is often cleaved which explains the reduced molecular mass peak of the hProCA32 conjugated with 1.24 kDa and 0.710 kDa PEG reagents (Figure 5.2 D and Figure 5.2 E). The next step is to
measure the relaxivity, metal binding and metal selectivity to conclude which length of PEG is suitable for \textit{in vivo} studies.
Figure 5.1 Iodine stained and coomassie blue stained gels depicting the PEGylation of hCA32.cys.

(A) The iodine stain and SDS-PAGE gel of hProCA32.cys, PEGylated with 10 kDa PEG. A 10kDa increase in the molecular size is clearly observed by the gels. Both staining suggest that the protein has been PEGylated with more than 90% efficiency.

(B) The iodine stain and SDS-PAGE gel of hCA32.cys, PEGylated with 2 kDa PEG. A 2kDa increase in the molecular size is clearly seen by the gels. In SDS-PAGE, a light density band is visible at 12kDa suggesting that some protein remained un-PEGylated.
Figure 5.2 the mass spectra of hProCA32.cys before and after PEGylation.

(A) MALDI spectrum of hProCA32.cys before PEGylation showing the molecular mass of 12.4 kDa. (B) hProCA32 after conjugation with 2 kDa PEG and peak centered at 14.43 kDa and (C) The zoomed in region of spectrum in B. Polydispersed peaks of mal.PEG reagent with 44.05 Da (one PEG units) difference between each peak is causing such peak broadening. (D) Mass spectrum with a peak at 13.34 kDa corresponding to hProCA32 conjugated with 1.24 kDa PEG and (E) Mass spectrum with a peak at 12.82 kDa corresponding to hProCA32 conjugated with 0.710 kDa PEG
5.2.2. Relaxivity measurements

The relaxivity of hCA32 was measured in 10 mM HEPES at pH 7.2, 37 °C. The Gd$^{3+}$ concentration was fixed at 100 µM and various hProCA32 concentrations were added from 0 to 200 µM. $T_1$ and $T_2$ of water for each sample were measured. It has been proposed that increasing the metal binding site shortens the transverse and longitudinal relaxation times and hence reducing the relaxivity. As seen in Figure 5.3, the relaxation times, $T_1$ and $T_2$ shorten as the ratio of protein to gadolinium increases until the concentration of protein reaches a 1:2 ratio with gadolinium. Keeping the gadolinium concentration fixed at 100 µM, the relaxation times decrease to the lowest at 50 µM of hProCA32, indicating 1:2 binding. The average relaxivity calculated at 1:2 ratio is indeed the highest, $r_1 = 27$ mM$^{-1}$ s$^{-1}$ and $r_2 = 36$ mM$^{-1}$ s$^{-1}$ and is comparable with the rat ProCA32 (Figure 5.4). The relaxivity value rapidly drops at the concentrations higher than 1:2 ratios (Figure 5.4). The reason behind this phenomenon is unclear and still needs to be explored. hProCA32 is known to have heterogeneous gadolinium binding sites. The S56D mutation in hProCA32 (The first residue methionine excluded) is identical to the S55D mutation in the wild-type α and β parvalbumin (The First residue methionine included) studied by Henzl et al[69]. The calcium-binding studies performed by Henzl reported the macroscopic stepwise binding constant for S55D mutants examined in HEPES-buffered saline at pH 7.4 at 25 °C where (CD site) $K_1 = 2.5(0.2) \times 10^8$ and (EF site) $K_2 = 6.2 (0.2) \times 10^7$[69]. Considering the similarities between the ionic radii and coordination of gadolinium and calcium it can be assumed that the gadolinium will exhibit similar trend in the binding affinities for each site. If we assume that the CD site has a stronger gadolinium binding affinity and EF site has a weaker gadolinium binding affinity, the overall average binding affinity still remains high. The
increase in relaxation times at concentrations below 50 μM of the protein, which is relatively lower than the gadolinium concentration, is mostly due to the saturation of both strong and weak metal binding sites. Once the protein concentration reaches above 50 μM, the gadolinium can selectively bind to the strong metal binding site leaving the weak metal binding site unoccupied. Hence, an increase in protein concentration causes a decrease in the amount of metal bound and thus reducing the relaxivity. Furthermore, the relaxivity studies of hProCA32 linked with cysteine PEG needs to be analyzed as a future direction.

Figure 5.3 The measurement of $T_1$ and $T_2$ relaxation time for hProCA32 with 100 μM Gd$^{3+}$
Figure 5.4 The relaxivity measurements of hCA32 compared with rat ProCA32 expressed by fermentation.

(A) $r_1$ and (B) $r_2$ relaxivities of rat ProCA32 and hProCA32 in buffer containing 10 mM HEPES, pH 7.2 and 100 μM of Gd$^{3+}$ at 37 °C.

5.2.3. Equilibrium Calcium titration

Humanized ProCA32 is originally a calcium-binding protein, α-parvalbumin with two metal binding pockets. α-Parvalbumin is reported to have a strong calcium binding ($K_d$ of around $10^{-9}$ M) [70]. Although some mutations were performed on PV to improve its Gd$^{3+}$ binding affinity, the possibility of binding to calcium remains highly likely. Hence, the calcium binding affinity of hProCA32 must be tested. However, it is difficult to control the free metal ion concentrations and measure strong affinity using direct fluorescence titration. To overcome these limitations, the Yang lab has developed metal chelator buffer system, where the free metals in
the system are tightly controlled by the high concentration of chelator, EGTA in this case. The total Ca²⁺ concentration in each titration point is tightly controlled, and the exact total Ca²⁺ concentrations were titrated into the system. For example, if 100 μM of total Ca²⁺ is titrated in the system, it generates 7.51 x 10⁻⁹ M free Ca²⁺ in the system according to Equation 5-1. The experiment was performed following the procedure in section 2.11 in which 1 μM of hProCA32 was added to buffer containing 5 mM EGTA, 50 mM HEPES, and 150 mM NaCl, pH 7.2 and calcium was titrated in increments, while monitoring the fluorescence with excitation at 280 nm and emission range of 300-400 nm. Calcium was titrated until the chelator present in the system (EGTA) was saturated; hence approximately 5 mM of total calcium was titrated into the system. The fluorescence changes of tryptophan are monitored to probe the changes caused by binding of calcium. Fluorescence intensities were graphed versus emission wavelength (Figure 5.5 A) and the maximum intensities at 311-315 nm were fitted by Hill equation (Equation 5-2) to obtain the Kd (Figure 5.5 B). The binding affinity of hProCA32 to Ca²⁺ was thus experimentally measured to be 1.35 x 10⁻⁸ M, which falls in the reasonable range and relatively comparable to the one for rat ProCA32.

\[
[Ca^{2+}]_{\text{free}} = Kd_{\text{Ca-EGTA}} \times \frac{[\text{Ca-EGTA}]}{[\text{EGTA}]_{\text{free}}}
\]

\[
f = \frac{[Ca^{2+}]_{\text{free}}^n}{Kd_{\text{Ca-EGTA}}^n+[Ca^{2+}]_{\text{free}}^n}
\]

Equation 5-1

Equation 5-2
Figure 5.5 Determining the metal binding affinity of hProCA32 using metal chelator buffer system.

(A) The fluorescence spectra of hProCA32-Ca$^{2+}$ titration using metal chelator buffer system with excitation at 280 nm and emission range of 300-400 nm. (B) The fitted curve of the maximum intensity at 311 nm with a dissociation constant of 1.35 × 10$^{-8}$ M using hill equation (Equation 5-2).

### 5.2.4. Determining metal binding affinity of hProCA32 using Tb$^{3+}$ EGTA/DTPA buffer system

Lanthanides tend to bind to EF-hand calcium-binding proteins with high affinity. Terbium (Tb$^{3+}$) is a trivalent lanthanide possessing similar ionic radius and coordination as calcium. Due to its unique fluorescence properties, Tb$^{3+}$ is frequently used as a probe in fluorescence spectroscopy to study the intrinsic metal binding properties of biomolecules. In FRET, accessible tryptophan upon excitation transfers energy to terbium and generates fluorescence signal. Tryptophan is excited at a wavelength of 282 nm, which emits at 340 nm; a wavelength range where terbium can absorb energy. Once the terbium absorbs energy at 340 nm,
it emits the energy between 545 nm and 595 nm. Our previously developed rat ProCA32 has been reported to have a strong binding affinity for Tb$^{3+}$ of about $10^{-22}$ M. The humanized ProCA32 must have a similar binding affinity to Tb$^{3+}$. However, it is extremely difficult to accurately measure the binding affinity in such small range using fluorescence. Fluorescence titration has limited sensitivity and the free metal in the solution can generate its own fluorescence. In order to overcome these limitations, metal chelator buffer system can be used to control the free metal in the system. The dissociation constants for chelators such as EGTA and DTPA to Tb$^{3+}$ are obtained from the National Institute of Standards and Technology (NIST). Changes in Trp fluorescence can be monitored to analyze the metal binding affinities at extremely low concentrations of free Tb$^{3+}$. The Tryptophan (Trp) residue present near the binding site can be excited at 280 nm, which has an emission at 315 nm. This can further excite the Tb$^{3+}$ that is bound to the protein, and the fluorescence emission can be observed at 545 nm.

This system contains 50 mM HEPES, 150 mM NaCl, 30 μM hProCA32, 5mM EGTA or DTPA and 0.1 μL Rhod-5N, pH7.2. EGTA and DTPA have a $K_d$ to Tb$^{3+}$ of $10^{-18}$ M and $9.55 \times 10^{-22}$ M, respectively. The system contains about 170 times higher concentration of the chelator than that of the protein; therefore the majority of the protein should be in its apo form (metal free form). Tb-EGTA system can create a buffer range from $10^{-18}$ to $10^{-9}$ M of free Tb$^{3+}$. EGTA is a weak chelator as compared to DTPA and can be used Tb-DTPA can generate a buffer range between $10^{-22}$ and $10^{-18}$ M of free Tb$^{3+}$. The $K_d$ of the protein is first tested in the weaker buffer range using EGTA followed by, the stronger buffer range using DTPA. As a weak metal binding indicator, the fluorescence of Rhod-5N can increase when free [Tb$^{3+}$] is higher than $10^{-6}$ M. Tb$^{3+}$ itself has its own fluorescence, thus as a background control, a parallel experiment was performed where 5 mM EGTA or DTPA, 50 mM HEPES, 150 mM NaCl pH 7.2 were used as a
buffer and Tb$^{3+}$ was titrated to the system.

**Figure 5.6 A** and **Figure 5.7 B** are the control experiments with Buffer- 5mM EGTA and buffer- 5mM DTPA, respectively. Various volumes of Tb$^{3+}$ were titrated into the system. B and D are the titration plots with 30 µM of hProCA32 into buffer-EGTA and Buffer-DTPA, respectively with fluorescence intensity subtracted from the background Tb$^{3+}$ from the control experiment. Various volumes of Tb$^{3+}$ were titrated in to the system, as the [Tb$^{3+}$] increased the fluorescence intensity at 545 nm, which is an indication of Tb$^{3+}$ binding to hProCa32.

The increase in fluorescence intensities at 545 nm in **Figure 5.6** and **Figure 5.7** demonstrates that energy transfer between Tb$^{3+}$ and hProCA32 occurred in both Tb-EGTA and Tb-DTPA buffer systems. Therefore, hProCA32 can bind to Tb$^{3+}$ at a low concentration range of $10^{-18}$ to $10^{-22}$ M. The fluorescence intensities at 545 nm were plotted using hill curve fitting, and binding affinity constants were calculated using Hill equation. hProCA32 has the $K_{d}^{hCA32,Tb}$ of $1.41 \times 10^{-18}$ M using Tb-EGTA buffer system and $K_{d}^{hCA32,Tb}$ of $7.79 \times 10^{-22}$ M using Tb-DTPA buffer system. These dissociation constants are comparable to the ones for rat ProCA32.
Figure 5.6 Fluorescence spectra of hProCA32 binding with Tb^{3+} using metal chelator buffer system.

(A) The EGTA buffer system contained 50 mM HEPES, 150 mM NaCl at pH 7.2, and 5 mM EGTA was used a control where (B) contained 50 mM HEPES, 150 mM NaCl at pH 7.2, 5 mM EGTA and 30 μM hProCA32. As the Tb^{3+} was titrated in to the system, the fluorescence intensity at 545 increased due to the binding of protein to the free metal. Once the total metal concentration is higher than the DTPA, low affinity dye Rhod-5N binds to the remaining metal causing an increase in fluorescence intensity at 575 nm, which indicates that the system has reached its saturation point. The curve has fluorescence intensities subtracted with the background signal generated by Tb^{3+} itself. The curve fitting of these intensities at 545 were done using Hillequation. (C) The actual fluorescence intensities plotted to monitor the dynamic range and (D) the normalized fluorescence intensities to obtain the binding affinities.
Figure 5.7 Determining Tb $^{3+}$ binding affinity of hProCA32 using Tb$^{3+}$-EGTA / DTPA system

(A) The EGTA buffer system contains 50 mM HEPES, 150 mM NaCl at pH7.2, 5 mM EGTA and 30 μM hProCA32. (C) Saturation test where the maximum fluorescence intensity peak corresponds to Rhod-5N-Tb$^{3+}$ complex where the minimum fluorescence intensity peaks corresponds to Rhod-5N ($\lambda_{Ex} = 551$ nm, $\lambda_{Em} = 576$ nm).
5.2.5. Determining the affinity of hProCA32 to Gd\(^{3+}\) using various fluorescence assays

The binding affinity of hCA32-Gd\(^{3+}\) was first determined using fluorescent dye, Fluo-5N pentapotassium salt, which is a weak calcium indicator. The binding affinity of Fluo-5N to Gd\(^{3+}\), 3.8 ± 0.2 \times 10\(^{-12}\) has been previously determined by Yang’s lab using Fluo-5N-NTA buffer system [29]. The experiment was performed using the lower metal and dye concentrations, 1 μM Fluo-5N and 1 μM Gd\(^{3+}\), in 10 mM HEPES at pH 7.2, and the initial fluorescence intensity was measured between 500 nm to 600 nm with excitation at 488 nm. Using this method, various concentrations of gadolinium were titrated gradually causing a decrease in fluorescence intensity as the saturation point approached. This decrease is caused since the contrast agent competes the gadolinium out from the dye and hProCA32-Gd\(^{3+}\) complex does not have a fluorescence of its own. This experiment was performed in triplicate. Fluorescence intensity versus wavelength is shown in Figure 5.8A. The fitted curve at maxima 519 nm displaying the \(K_{app}\) is shown in Figure 5.8 B. The \(K_d\) for hProCA32-Gd\(^{3+}\) was calculated using Equation 5-3 (derivation shown in Section 2.11). The binding affinity of hProCA32 and Gd\(^{3+}\) using the Fluo-5N competition assay is 1.08 \times 10\(^{-11}\) M, which is lower than the Ca\(^{2+}\) binding constant of hProCA32 (1.35 x 10\(^{-8}\) M).

\[
K_{d2} = K_{app} \frac{K_d}{K_{d1} + [Fluo-5N]_{Total}} \tag{Equation 5-3}
\]

Although this method is a reliable way to determine the binding affinity of our contrast agent, it has its own limitations, as Fluo-5N is a weak calcium indicator and its affinity for Gd\(^{3+}\) is in a lower range to begin with. Yang lab has developed another indirect technique utilizing the
Förster resonance energy transfer (FRET) property of Tb$^{3+}$ and tryptophan residue present in the protein. When excited at 280 nm, tryptophan residue present in hProProCA32 has an emission at 345 nm. This energy released by the tryptophan (donor) is transferred to the Tb$^{3+}$ (acceptor) that is present within the Förster’s distance at about 10 Å. Thus, Tb$^{3+}$ is excited at 315 nm and emits at 545 nm. In the competition assay, the protein was preloaded with Tb$^{3+}$ and Gd$^{3+}$ was titrated to the protein-Tb$^{3+}$ complex. Gd$^{3+}$ competes the Tb$^{3+}$ out of the metal binding pocket, and since there is no energy transfer between Gd$^{3+}$ and tryptophan, the emission at 545 nm decreases as Tb$^{3+}$ is competed out.

![Figure 5.8 Determining binding affinity of hProCA32 for Gadolinium using Fluo-5N competition assay](image)

\[ K_{app} = 2.84 \text{ uM} \]
\[ K_d = 1.08 \times 10^{-11} \text{ M} \]

Figure 5.8 Determining binding affinity of hProCA32 for Gadolinium using Fluo-5N competition assay

(A) Competition assay with 1 μM Fluo-5N and 1 μM gadolinium in 10 mM HEPES at pH 7.2 with hProCA32 titrated to determine the $K_d$ (excitation at 488 nm, emission 500-600 nm). (B) Fitted curve at maximum intensity of 519 nm displaying the $K_{app}=2.84$ μM and calculated $K_d$ of $1.08 \times 10^{-11}$ M.
Now that it is confirmed that our contrast agent is capable of binding to gadolinium and has a strong binding affinity for \(\text{Tb}^{3+}\), as determined by metal chelator buffer system, the next step is to determine the gadolinium binding affinity with applying this information in designing the experiment. Since \(\text{Gd}^{3+}\) does not fluoresce upon excitation, we use an indirect assay. Gadolinium binding affinity for hProCA32 was determined using competition experiments with \(\text{Tb}^{3+}\), where \(\text{Gd}^{3+}\) competes with \(\text{Tb}^{3+}\) that is bound to hProCA32 causing a decrease in fluorescence intensity. Ten micromolar of hProCA32 and 20 \(\mu\text{M}\) of \(\text{TbCl}_3\) in 50 mM HEPES, 150 mM NaCl, pH 7.2 were incubated with different \(\text{Gd}^{3+}\) concentrations ranging from 0 to 1000 \(\mu\text{M}\) at room temperature overnight. The \(\text{Tb}^{3+}\) fluorescence energy transfer signal changes were measured by excitation at 280 nm and emission at 545 nm. As shown in Figure 5.9 A, free \(\text{Gd}^{3+}\) indeed competes \(\text{Tb}^{3+}\) out of the binding pockets of hProCA32 indicating a decrease in fluorescence intensity upon addition of \(\text{Gd}^{3+}\) with an apparent \(K_d\) (\(K_{\text{app}}\)) of 8.3 \(\mu\text{M}\). The \(\text{Gd}^{3+}\) binding affinity of hProCA32 calculated using one to one binding equation was \(2.83 \times 10^{-22}\) M (Equation 2-7). However, \(K_d\) using this technique is highly sensitive to the values used for \(K_d\) of hProCA32-Tb\(^{3+}\), which in turn is highly dependent on the dissociation constants of the chelator-Tb\(^{3+}\). The binding affinity of hProCA32 falls in the similar range as compared to rat ProCA32 suggesting that despite of 20 % difference in the protein sequence, hProCA32 has similar physical characteristics as ProCA32 as a contrast agent.
Figure 5.9 Determining the Gd$^{3+}$ binding affinity of hProCA32 using competition assay with Tb$^{3+}$

(A) The Fluorescence spectra of Tb$^{3+}$FRET where the signal decreases as Gd$^{3+}$ competes the terbium out of hProCA32. (B) The fluorescence intensities at 545 nm were plotted against the concentration of Gd$^{3+}$ fitted using the one-to-one binding Equation 2-5 $\lambda_{\text{Ex}} = 288$ nm, $\lambda_{\text{Em}} = 545$ nm.

5.2.6. Determining Zn$^{2+}$ binding affinity of hProCA32 using a FluoZin-1 competition assay

As discussed earlier, in chapter 3, zinc is another physiological metal that might be expected to bind to our contrast agent causing Gd$^{3+}$ to be released freely in the body. This can lead to lethal toxicity in normal physiological functioning. Hence, it is extremely important to evaluate the binding affinity of hProCA32 for Zn$^{2+}$. The experiment was performed using the
aforementioned procedure in Section 2.9. The zinc-binding affinity of hProCA32 was determined by competition assay with 1 μM Fluozin-1 and 1 μM of Zn$^{2+}$ in 10 mM HEPES pH 7.2. As the contrast agent was titrated into the system, the fluorescence intensities decreased, indicating that the contrast agent was successful in grasping the zinc from the dye. The experiment was performed in triplicate and fluorescence intensity versus wavelength was graphed as shown in Figure 5.10 A. The fluorescence intensity maxima at 519-520 nm was averaged for three trials and used to fit the curve to obtain $K_{\text{app}}$ as shown in Figure 5.10 B. The $K_{\text{app}}$ for this competition assay, 2.07 μM, was used to calculate the $K_d$ using the following equation (derivation shown in Section 2.9):

$$K_{d2} = K_{\text{app}} \frac{K_{d1}}{K_{d1}+[\text{FluoZin-1}]_{\text{Total}}}$$  \hspace{1cm} \text{Equation 5-4}

The binding affinity of hProCA32 for Zn$^{2+}$ is 1.42 ±0.18 × 10$^{-6}$ M, which is comparable to the binding affinity of rat ProCA32 as shown in Table 5.1 in section 5.3.
Figure 5.10 Determining Zinc binding affinity of hCA32.

(A) Competition assay with 1 μM FluoZin-1 and 1 μM zinc in 10 mM Tris pH 7.2 and hProCA32 titrated in increasing concentration to determine the $K_d$, Excitation at 495 nm and Emission range : 500- 650 nm (Three trials) (B) Fitted curve with the maximum intensity at 520 nm versus the concentration of hProCA32 titrated in to the system

$$K_{app} = 2.07 \text{ uM}$$
$$K_d = 1.42 \pm 0.18 \times 10^{-6} \text{ M}$$

Figure 5.11 Determining Lutetium binding affinity of hProCA32.

$\lambda_{Ex}= 288 \text{ nm}, \lambda_{Em}= 546 \text{ nm}$

10 μM hProCA32, 20 μM Tb $^{3+}$ in 10 mM HEPES 150, mM NaCl, pH7.2

$$K_{d \text{Lu-hCA32}} = 4.23 \times 10^{-22} \text{ M}$$
5.2.7. Serum stability of hProCA32

The acceptability of a contrast agent depends on its metal stability and relaxivity. As seen in the earlier sections, hProCA32 demonstrates improved metal selectivity over other physiological metals such as Ca$^{2+}$ and Zn$^{2+}$ with high gadolinium binding affinity and relaxivity as compared to other commercially available contrast agents. Thus, with such remarkable qualities, hProCA32 can be a promising and potential MRI contrast agent. In order to achieve this goal, it is extremely important to test the in vivo stability of the contrast agent. Testing the stability implies on the in serum activity and its interactions with proteins and enzymes present under biological conditions. It is crucial for our contrast agent to maintain its intact structure and has no interactions with enzymes that can possibly cleave it. In order to study the stability of hProCA32 without PEGylation and improvement caused after PEGylation, 500 μM of both contrast agents were incubated at 37°C in 50% serum with twice the amount of gadolinium (1 mM) for 12 days. Samples were collected at various time points during the 12 days and examined via SDS-PAGE, Ponceau red staining and western blot (Figure 5.12, Figure 5.13 and Figure 5.14). hProCA32 remained stable in the serum for at least 8 hours as confirmed by SDS–PAGE, and Ponceau red staining of the membrane used for western blot. The cysteine PEGylated hProCA32 remained stable until 12 days where the lysine PEGylated hProCA32 remained stable until 8 hours. Western blot is sensitive and the antibodies used specifically binds to the protein and can be trapped on a film. However, the western blot shows unwanted serum bands as well. This can be justified with several possibilities: The contrast agent has some interactions with the proteins present in serum, the serum contains some proteins that can be identified by the antibodies, the improper washing of the membrane after adding secondary antibodies could be enhanced by the substrate added prior to filming the membrane.
Figure 5.12 The serum stability study of hCA32 at 37°C in presence of gadolinium.

(A) The Ponceau red staining of the membrane used for western blot, (B) The 15% SDS-PAGE and (C) The western blot corresponding to the membrane used for ponceau red staining for the samples before and after incubation in serum at 37°C.

The serum stability test performed on hProCA32 indicates that the protein is stable at least up to 8 hours after incubation. Rat ProCA32 is stable in serum until 12 days [39] and hProCA32 is also expected to show similar behavior. Many factors affect the outcome of such lengthy experiment such as the sample collection and preparation methods, in addition, the tubes

* Note: All the samples added to gel electrophoresis are of equal volume.
where the samples are stored should be tight enough to avoid any evaporation of the samples. The outcome of these experiments could be improved by increasing the amount of protein and optimizing the appropriate conditions for preparing the samples for SDS-PAGE.

**hCA32Cys-2kDa PEG**

Figure 5.13 The serum stability study of Cysteine PEGylated -hCA32 at 37°C in presence of gadolinium.

(A) The Ponceau red staining of the membrane used for western blot, (B) The 15 % SDS-PAGE and (C) The western blot corresponding to the membrane used for ponceau red staining for the samples before and after incubation in serum at 37°C
The sample for protein only has 3 bands, suggesting that some of the protein indeed PEGylated (Lane 3 in Figure 5.13 A and C). The bands at 12 kDa and 26 kDa are the monomer and dimer forms of the non PEGylated protein, respectively. Ponceau red staining and western blot confirm that the protein is stable until 12 days as clear bands are seen at around 15 kDa. Furthermore, SDS-PAGE shows that the protein degrades after 5 days of incubation; however, the samples collected on the following days show clear bands at 15 kDa until 12 days. This could have been due to an error in preparing that particular sample.
Figure 5.14 The serum stability study of Lysine PEGylated-hProCA32 at 37°C in presence of gadolinium.

Multiple bands between 12 kDa to 25 kDa are the bands corresponding to the protein PEGylated at multiple sites. The Ponceau red staining of the membrane prior to washing with antibodies suggests that the protein remained stable until 8 hours of incubation whereas the
western blot shows the protein remained stable until 3 days of incubation in serum at 37 °C in presence of gadolinium.

5.2.8. **Magnetic Resonance imaging of cysteine PEGylated hProCA32 and Future Directions**

In order to assess the ability of the protein as an imaging contrast agent with *in vivo* functioning, hProCA32.cys-2kDa cys-PEG was injected into mice implanted with a human cancer and MRI imaging performed by Dr. Shenghui Xue, using a 4.7 T Varian MRI scanner at Emory University. Since rat ProCA32 does not have any grafted targeting moiety, it is reported to enhance mostly the liver region [39]. Therefore, 5 mM of the contrast agent was injected via tail-vein injection into the mice, and MRI images were scanned at various time points using gradient echo and fast spin echo sequences. Gradient echo gives T1 weighted enhanced images whereas fast spin echo provides T2 weighted dark images. The T1 weighted images seen in Figure 5.15 show images before injection of contrast agent and after the injection of contrast agent. From these images, it can be concluded that the contrast agent dramatically enhanced the kidney and liver region after 15 minutes of injection. After 40 minutes of injection, the contrast agent seems to be distributing out of the kidney into the blood vessels. The kidney and liver region still shows some enhancements after 2 hours of injection. The contrast agent showed not only positive bright T1 weighted images but also demonstrated negative, dark T2 weighted images. As seen in Figure 5.16, the liver region is light before injection of the contrast agents, however after the injection of our contrast agent liver region appears even darker. Considering the positive preliminary enhancements, it can be concluded that hProCA32 not only fulfills essential requirement of being a contrast agent but also can definitely be a suitable candidate.
Figure 5.15 T1 weighted- Gradient echo sequenced MRI images of kidney and Liver before and after injection of hProCA32.cys-2kDa PEG.cys into the mice via tail vein injection,
Figure 5.16  T1 weighted- Fast spin echo sequenced MRI images of Liver before and after injection of hProCA32.cys-2kDa PEG.cys into the mice via tail vein injection.
5.3. Conclusion and Future directions

In this chapter, the overall biophysical properties of hProCA32 as a contrast agent have been investigated. The site-specific conjugation of hProCA32 to cys-PEG reagent at C-terminal cysteine has been successfully attained using various lengths of PEGs. The gel electrophoresis results confirm that the contrast agent has been conjugated with PEG with more than 90% efficiency. The PEGylated contrast agents are further characterized by its biophysical properties and functioning. The average $r_1$ and $r_2$ relaxivity of hProCA32 are 27 mM$^{-1}$ s$^{-1}$ and 36 mM$^{-1}$ s$^{-1}$, respectively, which are close to rat ProCA32 with $r_1$ and $r_2$ of 30 mM$^{-1}$ s$^{-1}$ and 40 mM$^{-1}$ s$^{-1}$, respectively[39]. The rat ProCA32 expressed in large culture volume by fermenter has $r_1$ and $r_2$ relaxivities of 24 mM$^{-1}$ s$^{-1}$ and 33 mM$^{-1}$ s$^{-1}$(Figure 5.4A and B). To provide more concrete evidence for our hypothesis “conjugation with PEG causes an increase in relaxivity”, further investigation is required.

The metal binding affinities of hProCA32 to Tb$^{3+}$ and Ca$^{2+}$ were tested using metal chelator buffer system to tightly control the free metal in the system. The Gd$^{3+}$ binding affinity of hProCA32 determined by competition assay with Tb$^{3+}$ was about 10$^{-22}$ M, which is comparable with Tb$^{3+}$-DTPA. In overall, the Gd$^{3+}$ binding affinity of hProCA32 was larger than Zn$^{2+}$ and Ca$^{2+}$ binding affinities (Figure 5.5, Figure 5.9 and Figure 5.10). These data are comparable with rat ProCA32 (Table 5-1). In terms of metal selectivity, Log ($K_{Gd}/K_{Ca}$) of gadolinium over zinc and calcium is significantly higher as well (Table 5-1). This suggests that hProCA32 will bind to Gd$^{3+}$ with significantly higher affinity than to the physiological metals such as zinc and calcium. Contrast agent should bind to Gd$^{3+}$ strongly to reduce toxic effects generated by the accumulation of free gadolinium in the body especially kidney.
Furthermore, the human serum stability of hProCA32 and PEGylated hProCA32 at 37°C showed that non-PEGylated hProCA32 is stable at least 8 hours after incubation. The conjugation of PEG indeed improved the stability of our contrast agent up to 12 days whereas; the lysine PEG conjugation improved the stability up to 3 days confirmed by Western blot. For more concrete conclusion, further optimization of experimental conditions is required. Subsequently, to establish the \textit{in vivo} functionality of hProCA32 as a contrast agent, gradient echo sequence (T1 weighted) and fast spin echo sequence (T2 weighted) of MRI images were taken using 4.7 T MRI Varian scanner. Gradient echo sequence suggests that our contrast agent clearly shows enhancements in kidney and liver after 15 minutes of injection. In summary, hProCA32 has high metal affinity, selectivity and stability with 10 times higher relaxivity as compared to the commercial contrast agent, Gd-DTPA.
Table 5.1 The summary of the $K_d$ values of hProCA32 to Tb$^{3+}$, Gd$^{3+}$, Ca$^{2+}$ and Zn$^{2+}$ using various metal binding assays such as metal chelator buffer system (Tb$^{3+}$, Gd$^{3+}$, Ca$^{2+}$) and competition assay using molecular probe FluoZin-1 dye for Zn$^{2+}$.

<table>
<thead>
<tr>
<th>$K_d$ (M)</th>
<th>Gd-DTPA (M)</th>
<th>ProCA32 (M)</th>
<th>hProCA32 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb$^{3+}$</td>
<td>$9.55 \times 10^{-22}$</td>
<td>$1.21 \pm 0.33 \times 10^{-21}$</td>
<td>$2.48 \times 10^{-21}$</td>
</tr>
<tr>
<td>Gd$^{3+}$</td>
<td>$1.86 \times 10^{-21}$</td>
<td>$2.79 \pm 0.36 \times 10^{-22}$</td>
<td>$3.29 \pm 0.1 \times 10^{-22}$</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>$1.51 \times 10^{-10}$</td>
<td>$3.57 \pm 0.01 \times 10^{-9}$</td>
<td>$4.13 \times 10^{-8}$</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>$6.31 \times 10^{-19}$</td>
<td>$6.00 \pm 2.0 \times 10^{-8}$</td>
<td>$5.14 \pm 0.18 \times 10^{-6}$</td>
</tr>
<tr>
<td>Lu$^{3+}$</td>
<td>N/A</td>
<td>N/A</td>
<td>$4.23 \times 10^{-22}$</td>
</tr>
<tr>
<td>Log ($K_d$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log ($K_d$)</td>
<td>10.9</td>
<td>13.1</td>
<td>13.72</td>
</tr>
<tr>
<td>Log ($K_d$)</td>
<td>2.5</td>
<td>14.3</td>
<td>15.34</td>
</tr>
</tbody>
</table>

2 This dissociation constant was determined using DTPA-Tb$^{3+}$ buffer system in 10 mM HEPES, 150 mM NaCl, 0.1 μM Rhod-5N pH 7.2 and dependent on the binding affinity of the chelator-Tb$^{3+}$ Ex = 288 nm and Em = 545 nm
3 This dissociation constant was determined using a competition with Tb$^{3+}$ in 10 mM HEPES, 150 mM NaCl. Ex = 288 nm and Em = 545 nm
4 This dissociation constant was determined using DTPA-Ca$^{2+}$ buffer system in 10 mM HEPES, 150 mM NaCl, pH 7.2. Ex = 280 nm and Em = 311 nm
5 Dissociation constant determined using competition with FluoZin-1 (1 μM FluoZin-1 and 1 μM zinc in 10 mM Tris PH 7.2) Ex = 495 nm and Em = 516-520 nm
6. OVER ALL CONCLUSION AND SIGNIFICANCE OF THIS WORK

MRI is a powerful diagnostic tool that is non-invasive with deep tissue penetration. MRI can provide detailed information about tissues and organs without utilizing any harmful radiations. Due to low sensitivity, contrast agents are mostly used to enhance MRI images. In the past decade, Dr. Jenny J. Yang’s lab has adopted and merged the idea of MRI contrast agents with excellent knowledge of protein chemistry and developed novel class of Protein based MRI contrast agents (ProCA). ProCAs can not only improve the low sensitivity with their dramatic contrasting ability but also target the specific cancer biomarkers for early detection of cancerous tissues using MRI. The first generation of ProCA was developed by constructing a Gd\(^{3+}\) binding site on a stable scaffold protein, domain 1 of rat CD2. ProCA1 demonstrated 10 times higher relaxivities as compared to the clinically used MRI contrast agents. High relaxivity, directly corresponds to the use of low dosage, and low toxicity. Furthermore, targeted ProCA1 proved to be excellent molecular imaging probe against breast cancer. During this process, a series of ProCAs were developed with even more improved *in vivo* properties.

ProCA32 containing two metal binding sites is thermo-stable and has excellent solubility and most importantly the production process is relatively easy. ProCA32 is known to accumulate in the liver when injected into mice and dramatically enhances the liver region when observed under MRI scanner. Thus, to further advance to the next stage of pre-clinical studies, rat based ProCA32 must be humanized to reduce the immunological effects when injected into the human body. The objective of this thesis is to optimize the production in the bacterial system and examine functional and biophysical properties of hProCA32. There are several questions need to be addressed for isolation of hProCA32 obtained from *E.coli* expression system. 1) What are the optimal expression conditions for the large-scale production of hProCA32?, 2) What are the
optimal conditions for the cost effective and time efficient purification of hProCA32 and how to isolate in its purest form under industrial set up?, 3) Does hProCA32 differ from rat ProCA32 in terms of its structural properties, metal binding affinities, metal selectivity, and stability and relaxation properties as a MRI contrast agent?, 4) How to reduce the possible in vivo immunogenicity effects generated by our contrast agent? 5) Can modification of hProCA32 with PEG improve it’s in vivo stability, solubility, bio-distribution and biocompatibility without disrupting the functioning of hProCA32? 6) Can site-specific cysteine PEGylation be applied to hProCA32 to attain homogeneity? 7) How different is hProCA32 from the cysteine PEGylated hProCA32 and which length of PEG is best suitable for the in vivo application with altering its metal-binding properties?

One of the major findings in this thesis is that hProCA32 and its variants can be successfully expressed and purified using the bacterial system. The optimal expression can be achieved using both BL21 (DE3) and BL21 (DE3).pLySs competent cells at reduced temperatures after induction. In purification process, boiling the soluble hProCA32 supernatant indeed showed the removal of major unwanted proteins. Furthermore, the protein was successfully isolated from nucleic acids using 3 % streptomycin and affinity column with a maximum final yield of about 32 mgL⁻¹. In addition, hProCA32 can be isotopically labeled with ¹⁵N using M9 minimal medium. Labeled h ProCA32 with ¹⁵N can be used for lanthanide binding studies using NMR as a future direction.

Another major finding is for metal binding studies, which suggests that hProCA32 has high binding affinity for Gd³⁺ with dissociation constant of 10⁻²² M as compared to the other physiological metals such as calcium (10⁻⁸ M) and zinc (10⁻⁶ M). The metal selectivity Log (K_{Gd}/K_{Ca}) of hProCA32 for Gd³⁺ is higher over calcium (Log (K_{Gd}/K_{Ca}) = 13.72) and zinc (Log
Various fluorescence metal binding assays were tested to develop an indirect metal binding assay. The relaxivity remained relatively comparable with rat ProCA32 with $r_1$ of 27 mM$^{-1}$s$^{-1}$ and $r_2$ of 36 mM$^{-1}$s$^{-1}$. Cysteine PEGylated hProCA32 showed excellent serum stability at 37 °C up to 12 days. However, these are preliminary results and must be repeated to draw any concrete conclusions. Furthermore, 2 K cysteine PEGylation of hProCA32 showed T1 weighted enhancements and T2 weighted properties when introduced into mice under MRI scanner. Another finding, suggests that hProCA32 can bind to trivalent Lu$^{3+}$ with a dissociation constant of $10^{-22}$ M, which proposes a possible application for our contrast agent in SPECT.

All these significant findings are supportive of hProCA32’s functional similarities with rat ProCA32 and it is evident that hProCA32 can prove to be a great candidate for future molecular targeting. The long-term goal is to optimize the stability by using suitable PEG length, which does not disrupt the physical properties and enhances the relaxation properties of hProCA32. Additionally, the binding ability of hProCA32 with other metals such as Gallium, Yttrium, Zirconium and Indium can be tested as future direction for its application in nuclear medicine imaging modalities such as combined PET/MRI.
REFERENCE


39. Xue, S., Design of Novel Protein-based MRI Contrast agents with high relaxivity and stability for biomedical imaging, in Biology2013, Georgia State University, Atlanta, GA.


