

THE UNIVERSITY OF OKLAHOMA  
SCHOOL OF CHEMICAL, BIOLOGICAL AND MATERIALS ENGINEERING  
BIOENGINEERING CENTER

ATTACHMENT OF ANNEXIN V AND HORSERADISH PEROXIDASE TO  
SINGLE-WALLED CARBON NANOTUBES

A THESIS SUBMITTED TO  
THE UNDERGRADUTE FACULTY

In partial fulfillment of the requirements for the degree of  
Bachelor of Science at  
University of Coimbra, Portugal

BY  
LUÍS FILIPE FERREIRA NEVES  
Norman, Oklahoma  
2007

ADSORPTION OF ANNEXIN V AND HORSERADISH PEROXIDASE ON  
SINGLE-WALLED CARBON NANOTUBES

APPROVED BY:

A handwritten signature in black ink, appearing to read "Roger H", with a long horizontal flourish extending to the right.

Dr. Roger Harrison

THESIS COMMITTEE

COPYRIGHT

By

Luís Filipe Ferreira Neves

May 2007

## ACKNOWLEDGMENTS

An endeavor of this magnitude cannot be completed without the support of and encouragement from my advisor, Dr. Roger G. Harrison. I would like to express my sincere gratitude to him for offering his help and guidance throughout the year.

I would like to thank my Portuguese advisors, Dr. Isabel Lopes and Dr. Miguel Morgado, for their advice and support.

The work in this thesis was carried out with helpful contributions from some collaborators: Dr. Naveen R. Palwai, Dr. David E. Martyn, Dr. Yongqiang Tan and Dr. Daniel E. Resasco.

I would like to express my appreciation to the University of Oklahoma International Student Services and International Office of the University of Coimbra.

I would also like to express my appreciation to Dr. Naveen Palwai who helped me in the lab at the beginning of this internship.

I am so thankful for the support I received from all my family and friends.

Finally, my special thanks go to the Writing Center of the University of Oklahoma for their assistance with this dissertation.

## TABLE OF CONTENTS

<b>ACKNOWLEDGMENTS</b> .....	<b>iv</b>
<b>LIST OF TABLES</b> .....	<b>ix</b>
<b>LIST OF ILLUSTRATIONS</b> .....	<b>xi</b>
<b>LIST OF APPENDICES</b> .....	<b>xvii</b>
<b>ABBREVIATIONS</b> .....	<b>xix</b>
<b>ABSTRACT</b> .....	<b>xxi</b>
<b>RESUMO</b> .....	<b>xxii</b>
<b>CHAPTER</b>	
<b>I. INTRODUCTION AND LITERATURE REVIEW</b> .....	<b>1</b>
1. Goals of research and project overview .....	2
2. Cancer .....	8
3. Annexins .....	12
3.1. Annexin V .....	21
4. Phosphatidylserine .....	29
5. Single walled carbon nanotubes .....	35
6. Synthesis of annexin V .....	46
6.1. Recombinant DNA technology .....	48
6.2. Overview of protein expression .....	54
6.2.1. Plasmid construction .....	57
6.2.2. Cell transformation .....	74
6.2.3. Cell expression .....	76
6.2.4. Protein purification .....	85

## TABLE OF CONTENTS (Continued)

6.2.5. Protein determination .....	94
6.2.6. HRV 3C protease .....	95
7. Protein purity analysis – gel electrophoresis .....	96
7.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) .....	96
8. Freeze drying .....	98
9. Attachment of annexin V to SWNTs using EDC and NHS .....	99
<b>II. MATERIALS .....</b>	<b>101</b>
Bacterial strains and plasmids .....	102
Enzymes .....	102
DNA synthesis, purification and analysis .....	102
<i>E. coli</i> cell cultures media .....	102
Cell transformation .....	102
Protein purification .....	103
Freeze drying / lyophilization .....	103
SDS-PAGE .....	103
SWNT suspension preparation .....	103
Adsorption of HRP on SWNTs .....	104
Attachment of HRP to SWNTs using EDC and NHS .....	104
Adsorption of annexin V on SWNTs .....	104
Attachment of annexin V to SWNTs using EDC and NHS .....	104

## TABLE OF CONTENTS (Continued)

Binding of SWNT-annexin V on plastic-immobilized phosphatidyl serine .....	105
<b>III. METHODS .....</b>	<b>106</b>
Primer design for PCR and construction of annexin V gene .....	107
Construction of annexin V gene .....	107
Construction of expression plasmid pET-30 Ek/LIC-annexin V ...	107
Cell transformation .....	107
Expression of recombinant protein and cell lysis .....	111
Protein purification .....	112
SDS-PAGE gel preparation .....	116
Freeze drying / lyophilization .....	117
Preparation of the SWNT suspension using sodium cholate or low-viscosity sodium carboxymethylcellulose as a dispersion agent .....	119
Adsorption of horseradish peroxidase on SWNTs .....	121
Attachment of HRP to SWNTs using EDC and NHS .....	122
Adsorption of annexin V on SWNTs .....	124
Attachment of annexin V to SWNTs using EDC and NHS .....	124
Binding of SWNT-annexin V on plastic-immobilized phosphatidyl serine .....	125
<b>IV. RESULTS AND DISCUSSION .....</b>	<b>127</b>
Cloning, overexpression and purification of annexin V .....	128
SDS-PAGE .....	134

## TABLE OF CONTENTS (Continued)

Single walled carbon nanotubes .....	136
Adsorption of HRP on SWNTs .....	136
Adsorption of annexin V on SWNTs .....	143
Attachment of HRP to CMC-SWNTs using EDC and NHS .....	143
Attachment of annexin V to SWNT-CMC using EDC and NHS ...	150
Future studies .....	154
Conclusion .....	155
<b>LIST OF REFERENCES .....</b>	<b>156</b>
<b>APPENDIX .....</b>	<b>166</b>



## LIST OF TABLES

### CHAPTER

#### I. INTRODUCTION AND LITERATURE REVIEW

1. Nomenclature of the annexin family .....	14
2. Non-EF-hand calcium binding proteins .....	19
3. General properties of the annexin V .....	26
4. Fusion tags available for pET constructs .....	53
5. Overview of the pET system process .....	59
6. Biomolecules purified by affinity chromatography .....	89
7. Annexin V properties after purification and HRP properties .....	133

#### IV. RESULTS AND DISCUSSION

8. Enzyme activity and protein concentration results for HRP .....	139
9. SWNT concentrations, as measured by the absorbance at 800 nm .....	140
10. Volumes in the global experiment .....	143
11. Enzyme activity and protein concentration results .....	147
12. SWNT concentrations, as measured by the absorbance at 800 nm ...	148

## LIST OF TABLES (Continued)

13. Volumes in the global experiment .....	150
14. Protein concentration results .....	151
15. SWNT concentrations, as measured by the absorbance at 800 nm ...	152

### APPENDIX

A.1. LB media composition .....	166
A.2. LB Agar / SOC media composition (per Liter) .....	167
A.3. Kanamycin properties .....	168
A.4. Reagents required for the casting of mini-SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels .....	188
A.5. Marker molecular weights .....	190

## LIST OF ILLUSTRATIONS

### Chapter I: Introduction and Literature Review

1. Horseradish peroxidase structure .....	3
2. The injection of the complex SWNT-protein in the bloodstream .....	5
3. Positron emission tomography scanning .....	5
4. Overview of the experimental work .....	7
5. Age-adjusted cancer death rates, males by site, US, 1930-2002 .....	9
6. Age-adjusted cancer death rates, females by site, US, 1930-2002 .....	10
7. Leading sites of new cancer cases and deaths .....	11
8. Stereoviews of annexin V .....	16
9. Human annexin V structure .....	17
10. The canonical annexin structure .....	19
11. Annexin protein structure .....	20
12. <i>Homo sapiens</i> annexin V crystal structure .....	22
13. Human annexin V structure .....	23

## LIST OF ILLUSTRATIONS (Continued)

14. Molecular organization of phosphatidylserine (PS) .....	30
15. Schematic representation of annexin V illustrating the $\text{Ca}^{2+}$ - phospholipid binding affinity .....	32
16. General structure of a SWNT .....	36
17. $S_{11}$ absorption spectrum of a SWNT sample .....	40
18. Schematic diagram showing the global mechanism of this study .....	43
19. A single walled carbon nanotube wrapped with CMC-annexin V .....	45
20. Annexin V production general overview .....	47
21. Low-temperature electron micrograph of a cluster of <i>E. coli</i> bacteria ...	50
22. Separation principles in chromatography purification .....	55
23. A review of DNA technology and genetic engineering .....	56
24. Control elements of the pET system .....	61
25. 6x-His-Protein (N-terminus) schematic diagram .....	63
26. pET-30 Ek/LIC vector map .....	64
27. The pET-30 Ek/LIC vector map .....	64

## LIST OF ILLUSTRATIONS (Continued)

28. Ek/LIC overhangs .....	65
29. pET-30 Ek/LIC cloning/expression region .....	65
30. Overview of the cell transformation .....	67
31. Essential features of a useful vector such as the plasmid that encodes insulin .....	70
32. Restriction enzyme .....	72
33. Construction of a recombinant DNA molecule from two unrelated plasmids .....	73
34. Overview of cell transformation .....	75
35. Kanamycin .....	77
36. <i>E. coli</i> growth curve .....	79
37. The first line shows the genes of the <i>lac</i> operon, <i>lacZ</i> , <i>lacY</i> , and <i>lacA</i> . Transcription is repressed by the <i>lac</i> repressor .....	81
38. Isopropyl-beta-D-thiogalactopyranoside (IPTG) structure .....	83
39. An example of control of gene expression as exemplified by the <i>lac</i> operon .....	84

## LIST OF ILLUSTRATIONS (Continued)

40. Typical affinity purification .....	86
41. Step elution and gradient elution .....	87
42. Gradient elution of a (His) <sub>6</sub> fusion protein .....	87
43. Overview of a typical purification flow scheme for (histidine) <sub>6</sub> -tagged proteins using HisTrap HP including purification under denaturing conditions .....	90
44. Imidazole chemical structure and histidine chemical structure .....	92
45. HRV 3C protease experimental outline .....	95
46. Freeze drying .....	98
47. Carboxymethylcellulose structure .....	99
48. One-step EDC reaction with carboxyl and amine-containing molecules.	100

### Chapter III: Methods

49. Orbital shaker (Queque <sup>®</sup> Orbital Shaker) .....	109
50. Plate illustrating diagram .....	109

## LIST OF ILLUSTRATIONS (Continued)

51. Illustrating diagram to make a spreader .....	110
52. Plate illustrating diagram .....	110
53. Protein purification steps flow diagram .....	114
54. Overview of the chromatography system .....	115
55. Illustrated diagram of the freezing process .....	118
56. SWNTs suspended in sodium cholate .....	120

### Chapter IV: Results and Discussion

57. Step elution using affinity chromatography .....	130
58. Gradient elution of a (His) <sub>6</sub> fusion annexin V using affinity chromatography .....	131
59. Coomassie blue 12 % sodium dodecyl sulfate-polyacrylamide gel of the various purification steps .....	135
60. Enzyme activity assay .....	138
61. UV-Vis-NIR absorbance spectrum (normalized) .....	141
62. AFM images showing SWNT with sodium cholate and HRP protein ...	142

## LIST OF ILLUSTRATIONS (Continued)

63. HRP activity assay .....	145
64. HRP activity assay .....	145
65. Enzyme activity assay .....	146
66. UV-Vis absorbance spectrum .....	149
67. Absorbance at 800 nm vs. SWNT concentration plot .....	149
68. UV-Vis absorbance spectrum .....	153

## APPENDIX

A.1. Sonication process .....	172
A.2. Bradford protein assay .....	183
B.1. pET-30 Ek/LIC vector map showing the multiple cloning site .....	195



## LIST OF APPENDICES

Appendix	Page
A. Laboratory Protocols .....	166
A.1 Reagent Protocols .....	166
A.1.1 Luria-Bertani media .....	166
A.1.2 LB agar .....	167
A.1.3 Additives .....	168
A.1.4 Kanamycin preparation .....	169
A.1.5 IPTG preparation .....	170
A.1.6 Sonication buffer preparation .....	171
A.1.7 Sonication protocol .....	172
A.1.8 HRV 3C protease .....	173
A.1.9 Tris-HCl .....	174
A.1.10 Acrylamide (29%) Bis (1%) .....	175
A.1.11 Electrophoresis buffer .....	176
A.1.12 Ammonium persulfate preparation .....	177
A.2 Experimental Protocols .....	178
A.2.1 Transformation Protocol .....	178
A.2.2 Bradford protein assay .....	182
A.2.3 Enzymatic assay of peroxidase .....	184
A.2.4 SDS-PAGE analysis of proteins .....	188
A.2.5 Freeze dryer protocol .....	191
B. pET-30 Ek/LIC .....	194
C. pET-30 Ek/LIC sequence / Annexin V sequence .....	196
D. Annexin V protein structure .....	202

**LIST OF APPENDICES (Continued)**

<b>Appendix</b>	<b>Page</b>
E. EDC .....	203

## ABBREVIATIONS

**ABTS** – 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)

**AFM** – Atomic force microscopy

**Asp** – Aspartic acid

**BSA** – Bovine serum albumin

**CaBPs** – Calcium-binding proteins

**CD** – Circular dichroism

**CMC** – Carboxymethylcellulose

**dH<sub>2</sub>O** – Distilled water

**DNA** – Deoxyribonucleic acid

*E. coli* – *Escherichia coli*

**EDC** – 1-Ethyl-3-[3-dimethylamonopropyl] carbodiimide hydrochloride

**EF** – Edge-to-face

**Ek** – Enterokinase

**Fwhh** – Full width at the half-height

**His** – Histidine

**HRP** – Horseradish peroxidase

**IPTG** – Isopropyl-beta-D-thiogalactopyranoside

**Kan** – Kanamycin

**Kd** – Dissociation constant

**LB** – Luria-Bertani

**LIC** – Ligation-independent cloning

**Lys** – Lysine

**MES** – 2-(N-morpholino)ethanesulfonic acid

**mRNA** – Messenger ribonucleic acid

**MWNT** – Multiple walled carbon nanotubes

**NHS** – N-hydroxy-succinimide

**NIR** – Near-infrared

**PBS** – Phosphate buffer saline

**PCR** – Polymerase chain reaction  
**PE** – Phycoerythrin  
**PEG** – Poly(ethylene glycol)  
**PET** – Positron emission tomography  
**pH** – Potential hydrogen  
**PMSF** – Phenylmethylsulfonyl fluoride  
**PS** – Phosphatidylserine  
**RBS** – Ribosome binding site  
**RES** – Reticuloendothelial system  
**RNA** – Ribonucleic acid  
**ROS** – Reactive oxygen species  
**SDS** – Sodium dodecyl sulfate  
**SDS-PAGE** – Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
**SWNT** – Single walled carbon nanotube  
**Thr** – Thrombin  
**TPCK** – N- *p*-tosyl-L-phenylalanine chloromethyl ketone  
**Trp** – Tryptophan  
**US** – United States  
**UV** – Ultraviolet  
**Vis** – Visible  
**VTA** – Vascular targeting agent

# **ATTACHMENT OF ANNEXIN V AND HORSERADISH PEROXIDASE ON SINGLE-WALLED CARBON NANOTUBES**

LUÍS FILIPE FERREIRA NEVES

## **ABSTRACT**

To develop a therapeutic system with cancer cell selectivity, the present study proposes a possible specific and localized tumoral treatment. Phosphatidylserine exposure on the external face of the cell membrane is almost completely exclusive to cancer cells and endothelial cells in the tumor vasculature. With this knowledge and attending to the fact that the protein annexin V has excellent  $\text{Ca}^{2+}$ -phospholipid binding properties, we have excellent conditions for the development of a therapeutic system.

The synthesis of the protein annexin V, by a pET vector in *Escherichia coli*, constitutes the first phase of this study. Recombinant annexin V was purified from the cell lysate supernatant by metal affinity chromatography. The overall production of purified annexin V protein is 145 mg/L. A broad range of parameters such as SWNT- and PS-binding capacities were evaluated and optimized.

The attachment of annexin V to SWNTs will give a complex that can be used to target cancer cells and endothelial cells of the tumor vasculature that have PS exposed on their cell surface. This association makes possible cancer cell destruction, using localized irradiation provided by NIR-radiation. The energy absorption by the nanotubes provokes a temperature increase in the cancer cells culminating in the denaturation and loss of cellular functionalization. The specificity of treatment should result in minimal secondary effects to the normal cells. The binding of the complex SWNT-annexin V-PS can be evaluated using UV-Vis-NIR spectroscopy.

# LIGAÇÃO DE ANEXINA V E DE PEROXIDASE (ARMORÁCIA RUSTICANA) EM NANOTUBOS DE CARBONO DE PAREDE SIMPLES

LUÍS FILIPE FERREIRA NEVES

## RESUMO

Atendendo à necessidade do desenvolvimento de um sistema terapêutico com selectividade a células cancerígenas, neste estudo é apresentado um possível sistema de tratamento tumoral específico e localizado. A exposição de PS na face externa da membrana celular é quase exclusiva das células cancerígenas e das células endoteliais da vasculatura tumoral. Com este conhecimento e atendendo ao facto de que a proteína anexina V, apresenta excelentes propriedades de ligação  $\text{Ca}^{2+}$  - fosfolípidos temos excelentes condições para o desenvolvimento de um sistema terapêutico.

A síntese da proteína anexina V, recorrendo à utilização de um vector pET expresso em *Escherichia coli* constitui a primeira fase deste estudo. A proteína recombinante anexina V foi purificada a partir do sobrenadante através de cromatografia de afinidade metálica. A produção de anexina V pura é de 145 mg/L. Um variado conjunto de parâmetros, tais como as capacidades de ligação dos nanotubos de carbono de parede simples e fosfatidilserina foram avaliados e otimizados.

A ligação da anexina V aos nanotubos de carbono de parede simples origina um complexo que pode ser utilizado para a marcação selectiva de células cancerígenas e células endoteliais da vasculatura tumoral que apresenta fosfatidilserina exposta ao nível da superfície celular. Esta associação torna possível a destruição das células cancerígenas, recorrendo-se à irradiação localizada de radiação do infravermelho próximo. A absorção energética ao nível dos nanotubos, conduz a um aumento da temperatura ao nível das células cancerígenas que culmina na desnaturação e perda de funcionalização celular. A especificidade do tratamento resultará em escassos efeitos secundários para as células normais. A ligação do complexo SWNT-anexina V-fosfatidilserina pode ser avaliada recorrendo à utilização de espectroscopia UV-Vis-NIR.

# **CHAPTER I**

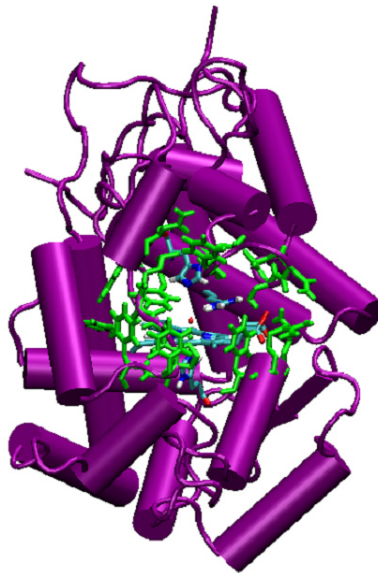
## **INTRODUCTION AND LITERATURE REVIEW**

## 1. Goals of research and project overview

The adsorption of annexin V on SWNTs was one of the main goals of this work. The protein adsorption on SWNTs has been studied and different strategies are available. In the current study, two different protocols were performed. These protocols were developed based on previous studies. Each protocol was initially tested using a model protein - horseradish peroxidase (HRP).

The protein HRP, a 40 kDa monomeric protein with a chain 308 amino acids long (Figure 1), was used in our study in order to perform the initial test studies, with the main goal of optimization of the protocols. This protein was used as a model protein because of the fact that it is cheap and can be easily purchased, while annexin V requires performing of several associated steps in our laboratory and its production is considered expensive. Because of HRP's characteristics, it can be considered a similar protein compared to annexin V. Another advantage associated with the use of HRP is the fact that it is possible to measure its activity (using an enzyme activity assay) and also its concentration (using Bradford protein assay).





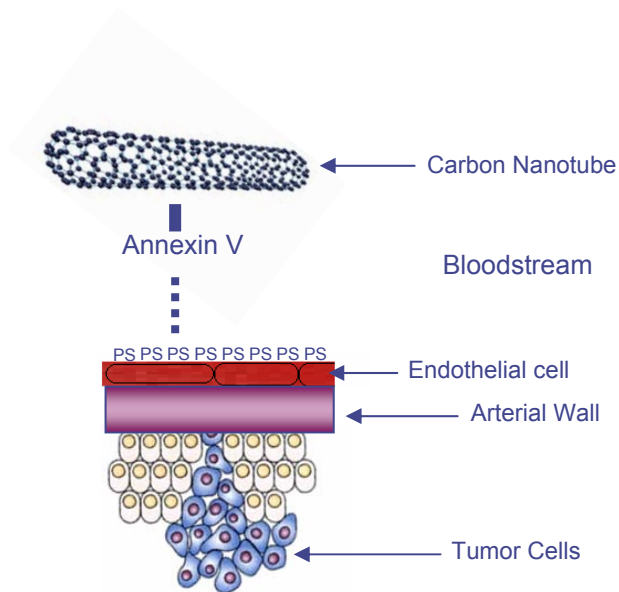
**Figure 1:** Horseradish peroxidase structure. Active sites are highlighted in green.  
(<http://publications.csail.mit.edu/abstracts/abstracts06/kathryn/hrp.jpg>)

The *in vivo* administration of the SWNT-CMC-annexin V complex (Figure 2) allows for the complex to selectively bind to PS exposed on the endothelial cells that line the tumor vasculature since annexin V selectively binds to PS. This specific selectivity makes possible the realization of studies of the identification and localization of metastasis (Sanchez 2004).

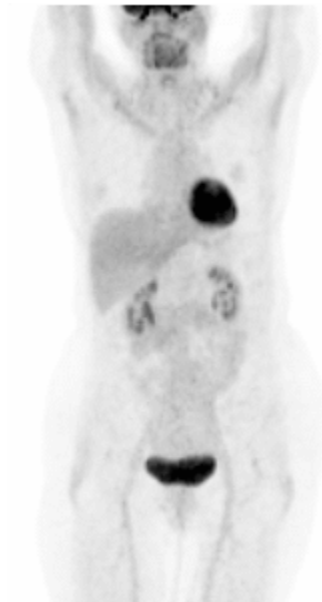
Because carbon nanotubes strongly absorb NIR energy, the irradiation with NIR of a specific region with an established connection between the SWNT complex and the cancer cells induces the nanotubes' energetic absorption causing a localized temperature increase. Consequently, this temperature increase contributes to the elimination of cancer cells.

Some studies demonstrate that the non-bound nanotubes have largely been eliminated through the excretory system (Liu and Wang 2007). There are high accumulation levels in the liver, bladder and spleen (Liu and Wang 2007), showing a good organism eliminating profile.

The use of this system, combined with the use of radioactive isotopes, (Tait, Smith et al. 2002; Liu and Wang 2007) allows for the realization of excellent imaging studies, using imaging techniques, such as, positron emission tomography (PET) (Figure 3) (Liu and Wang 2007).



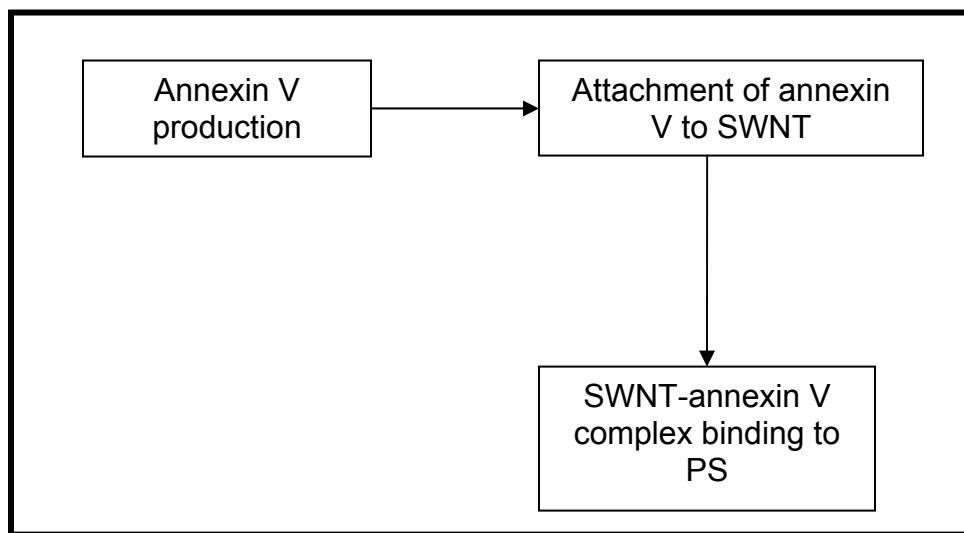
**Figure 2:** The injection of the complex SWNT-protein in the bloodstream allows a global and effective spread through the entire body, with an efficient phospholipid binding affinity ([http://science.nasa.gov/headlines/y2002/15jan\\_nano.htm](http://science.nasa.gov/headlines/y2002/15jan_nano.htm)).



**Figure 3:** Positron emission tomography scanning (<http://www.molecularimaging.vcu.edu/>).

The creation of a therapeutic system to treat the cancer is the main goal of this work. An overview of the experimental work for this project is shown in Figure 4. The binding of the SWNT-annexin V complex will be tested using plastic microtiter plates with PS immobilized.

Assuming the SWNT-annexin V complex binds selectively to microtiter plates with PS exposed, the complex will be tested for its ability to bind to human endothelial cells *in vitro* (in which PS has been induced to be exposed on the cell surface). After this, tests will be carried out using mice with tumor xenografts implanted, in which the SWNT-annexin V complex is injected i.v. and the tumor is exposed to NIR light.



**Figure 4:** Overview of the experimental work.

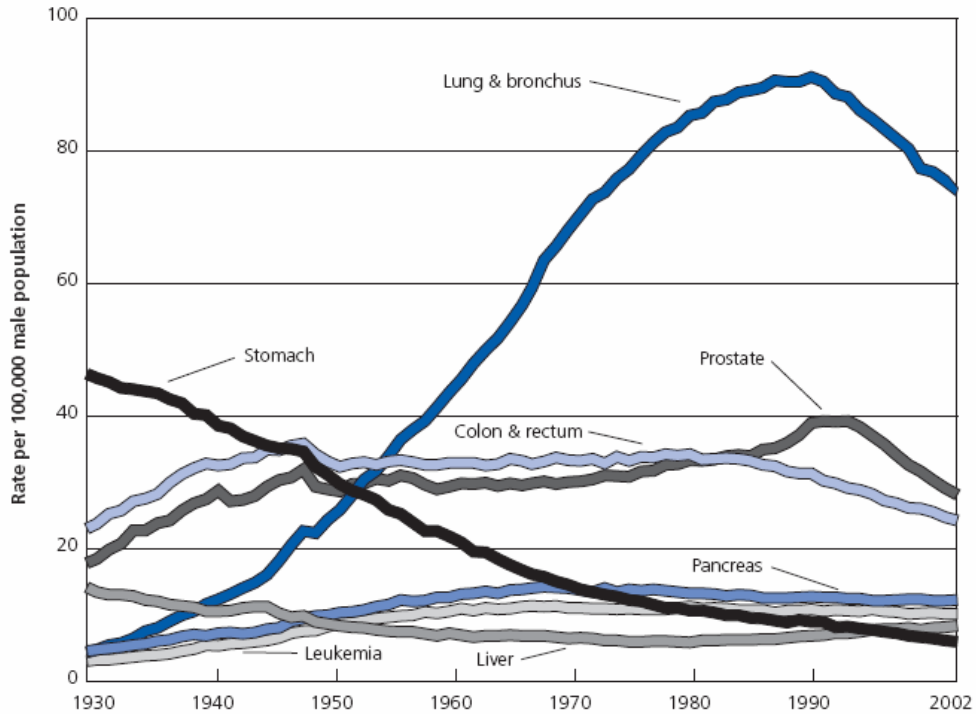
## 2. Cancer

Cancer can be defined as a group of diseases characterized by an uncontrolled cell division, invading the adjacent or distant tissues (metastasis) (Sanchez 2004). Being considered the world's most complex disease process, it is related to 100 or more single diseases (Fink 1979). There are several possible environmental factors that can induce cancer, such as exposure to radiation, hereditary factors, viruses, smoke, alcohol, dietetic habits, contributing to approximately 80 % of cancers (Fink 1979; Cooper 2004).

Cancer can exhibit different symptoms, although it can be asymptomatic until advanced stages. Risk factors include geography, ethnics, dietary habits, age, and hereditary genes that increase the predisposition for cancer.

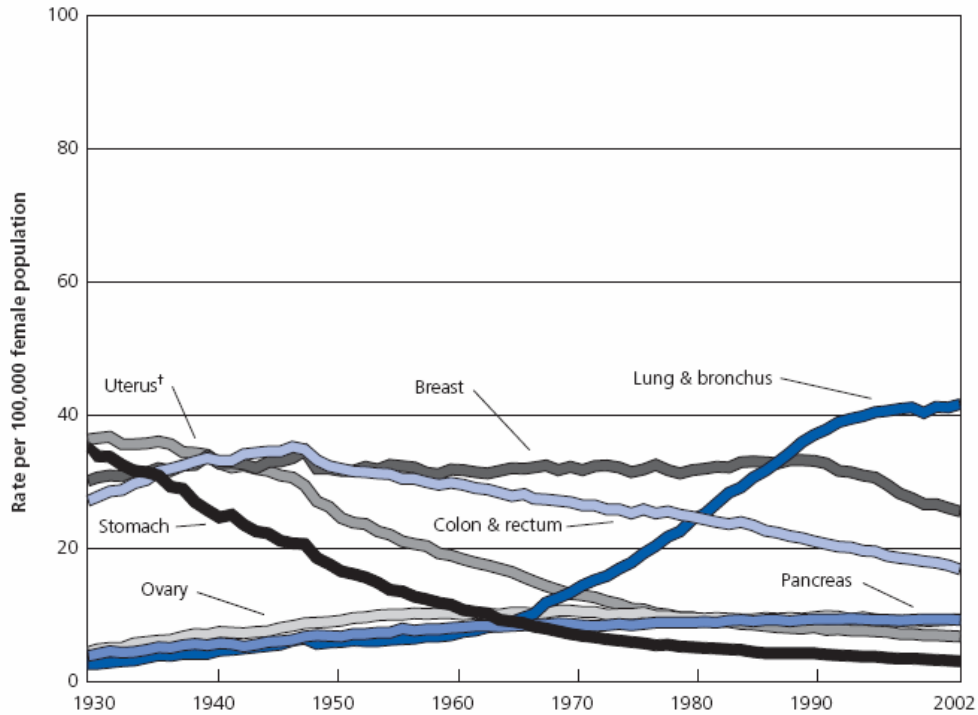
According to the statistics, the frequency of a specific cancer may depend on gender (Figures 5 and 6) (<http://www.medicinenet.com/>). According to the American Cancer Society 1,399,790 new cancer cases were expected to be diagnosed, excluding carcinoma *in situ* (noninvasive cancer) of any site (except urinary bladder) and basal and squamous cell skin cancers. Being considered the second leading cause of death in the United States of America, the statistics confirm that there are more than 1,500 persons dying per day as victims of cancer (American Cancer Society 2006). The most common type of cancer in the United States of America is the prostate and breast cancer (Figure 7).

Age-Adjusted Cancer Death Rates,\* Males by Site, US, 1930-2002



**Figure 5:** Age-Adjusted Cancer Death Rates\*, Males by Site, US, 1930-2002. \* Per 100,000, age-adjusted to the 2000 US standard population. Note: Due to changes in ICD coding, numerator information has changed over time. Rates for cancer of the liver, lung and bronchus, and colon and rectum are affected by these coding changes. Source: US Mortality Public Use Data Tapes 1960 to 2002, US Mortality Volumes 1930 to 1959, National Center for Health Statistics, Centers for Disease Control and Prevention, 2005. (American Cancer Society 2006)

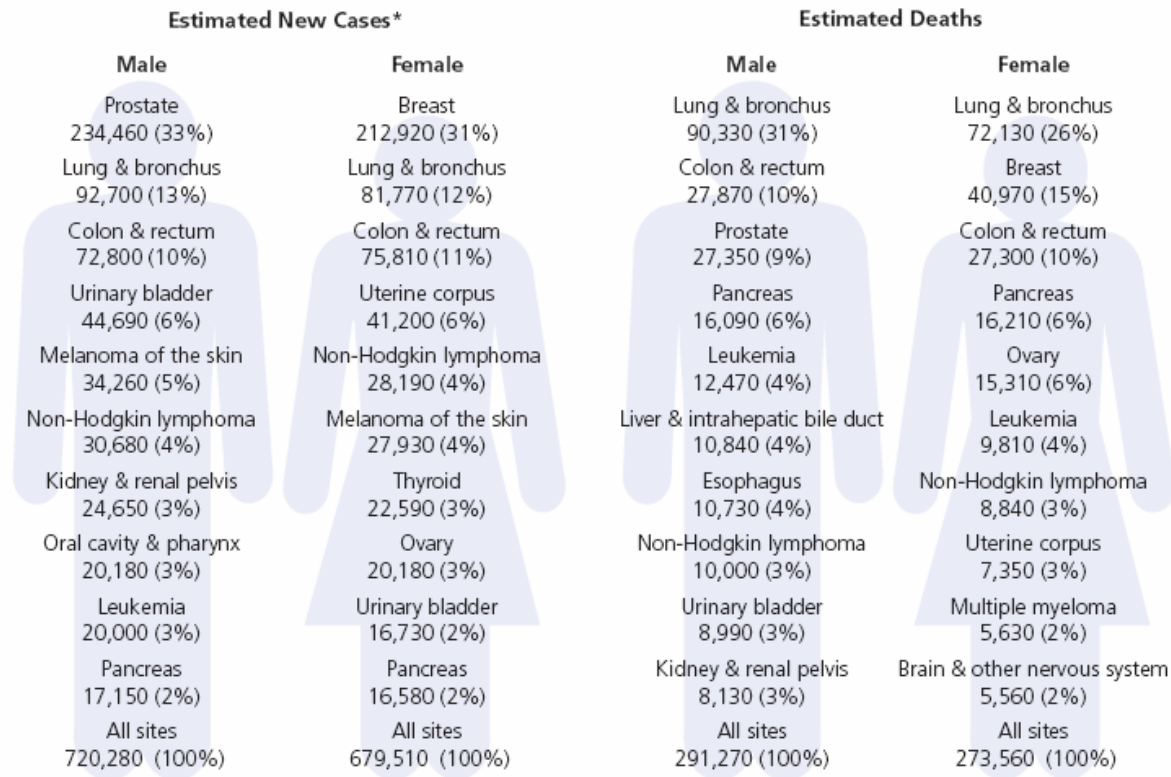
Age-Adjusted Cancer Death Rates,\* Females by Site, US, 1930-2002



**Figure 6:** Age-Adjusted Cancer Death Rates\*, Females by Site, US, 1930-2002. \* Per 100,000, age-adjusted to the 2000 US standard population. Note: Due to changes in ICD coding, numerator information has changed over time. Rates for cancer of the liver, lung and bronchus, and colon and rectum are affected by these coding changes. Source: US Mortality Public Use Data Tapes 1960 to 2002, US Mortality Volumes 1930 to 1959, National Center for Health Statistics, Centers for Disease Control and Prevention, 2005. (American Cancer Society 2006)



## Leading Sites of New Cancer Cases and Deaths – 2006 Estimates



**Figure 7:** Leading sites of new cancer cases and deaths. \*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder. Note: Percentages may not total 100% due to rounding (American Cancer Society 2006).

In order to execute cancer treatment, there is an inherent difficulty in distinguishing malignant cells from normal cells in the organism. Both of them share the same origin, which explains the fact that cancer cells are not significantly recognized by the immune system.

Generally, the treatment results from the combination of different therapeutic techniques, such as surgery and chemotherapy (Sanchez 2004). According to some studies, these techniques can be very efficient; however, they carry associated-secondary effects (Sanchez 2004) for the patient. A specific case is chemotherapy, a therapy based on the introduction of chemical substances in the bloodstream in order to control cell processes. The secondary effects associated with this therapy are related to the collateral destruction of normal cells (Sanchez 2004). When manifested, examples of these effects are anxiety, anemia and fatigue due to the red blood cells decreasing, diminution of white cells and platelets, renal perturbations, digestive system alterations and loss of appetite, increase in weight, alopecia, in addition to other symptoms.

Because of the need for an improved system to treat cancer, the general goal of this study is to kill all cancer cells in the organism, while not affecting the healthy cells. By specifically targeting cancer cells, the secondary effects associated with the conventional therapeutic methods are avoided. The development of a system with intravenous administration allows a global spread through the entire organism, eliminating the conventional problems of localization and treatment of metastatic regions (Sanchez 2004).

In this study, a protein is used that has PS-binding properties – annexin V – and is a very important marker for cancer cells, since that cancer cells and endothelial cells in the tumor vasculature have PS exposure on the external leaflet of the plasmatic membrane.

### 3. Annexins

The annexins are a group of proteins with similar properties (Smith, Kaetzel et al. 1990; Heizmann 1991) that were initially named by Geisow (Geisow 1986; Smith,

Kaetzel et al. 1990). That name is derived from the Greek *annex* meaning “bring/hold together” (Benz and Hofmann 1997). The annexins are different proteins, with a common C-terminus attached to an amino acid repeated domain, although presenting a unique N-terminal domain (Schlaepfer and Haigler 1990; Smith, Kaetzel et al. 1990; Heizmann 1991; Benz and Hofmann 1997) (Figures 8 and 9).

Discovered by Christopher J. Pazoles, Carl E. Creutz and Harvey B. Pollard approximately 27 years ago at the National Institute of Health (Bandorowicz-Pikula 2003), the annexins are a protein family (Table 1) comprised of 12 different proteins with a common important characteristic –  $\text{Ca}^{2+}$  - phospholipid binding affinity (Klee 1988; Schlaepfer and Haigler 1990; Smith, Kaetzel et al. 1990; Barton, Newman et al. 1991; Heizmann 1991; Celio 1996; Benz and Hofmann 1997). This discovery was the beginning of a promising science field which has had noticeable growth. These calcium-dependent membrane binding proteins (Huber, Romisch et al. 1990; Heizmann 1991) are involved in the membrane-related processes of exocytosis (Schlaepfer and Haigler 1990), adhesion, fusion and remodeling of the plasma membrane (Huber, Romisch et al. 1990; Sherbet 2000), phospholipase regulation, cytoskeletal organization (Schlaepfer and Haigler 1990), cell growth and transformation (Heizmann 1991). Adhesion mechanics, membrane traffic (Schlaepfer and Haigler 1990; Heizmann 1991; Celio 1996), signal transduction (Schlaepfer and Haigler 1990) and/or developmental process are other proposed functions (Crumpton 1992).

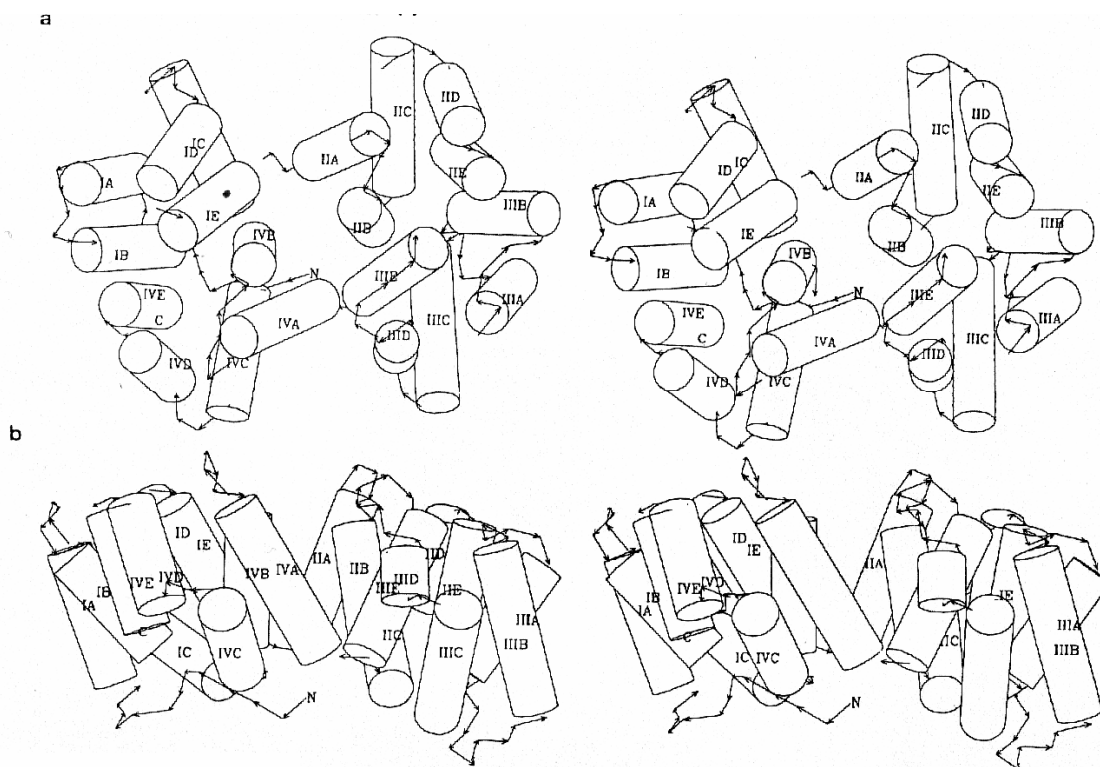
**Table 1:** Nomenclature of the annexin family (Crumpton 1992).

<b>Annexin</b>	<b>Synonyms</b>	<b>Annexin</b>	<b>Synonyms</b>
<b>I</b>	Lipocortin I Calpactin II P35 Chromobindin 9	<b>V</b>	Placental anticoagulant protein I Inhibitor of blood coagulation Lipocortin V 35 kDa calelectrin
<b>II</b>	Calpactin I heavy chain Lipocortin II P36 Chromobindin 8 Protein I Placental anticoagulant protein IV		Endonexin II Placental protein 4 Vascular anticoagulant- $\alpha$ 35- $\gamma$ calcimedin Calphobindin I Anchorin CII
<b>III</b>	Lipocortin III Placental anticoagulant protein III 35- $\alpha$ calcimedin	<b>VI</b>	p68, p70, 73k 67 kDa calelectrin Lipocortin VI Protein III Chromobindin 20 67 kDa calcimedin Calphobindin II
<b>IV</b>	Endonexin I Protein II 32.5 kDa calelectrin Lipocortin IV Chromobindin 4 Placental anticoagulant protein II Placental protein 4-X 35- $\beta$ calcimedin	<b>VII</b> <b>VIII</b> <b>IX and X</b> <b>XI</b> <b>XII</b>	Synexin Vascular anticoagulant- $\beta$ (only reported in Drosophila)  (only reported in Hydra)

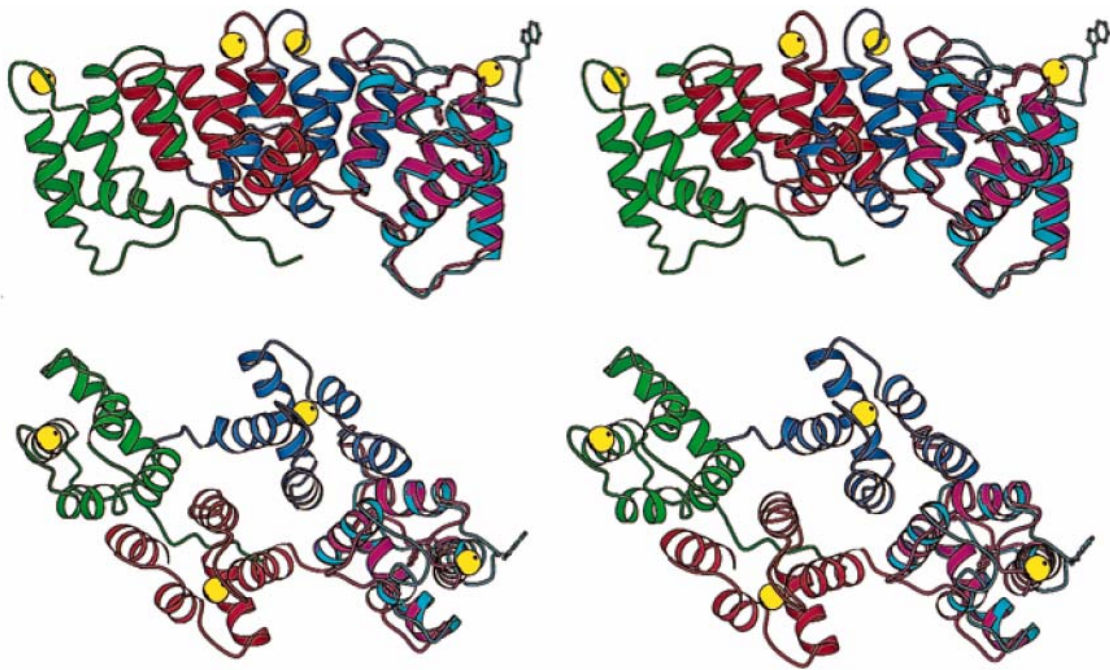
The recently cloned intestinal-specific annexin (ISA) is not included, but might tentatively be regarded as annexin XIII.

The annexins are a group of proteins that have been studied exhaustively regarding their important biochemical properties, although their specific function is still unclear (Huber, Romisch et al. 1990; Barton, Newman et al. 1991; Celio 1996). The annexins are present in higher and lower eukaryotes (Burger, Berendes et al. 1993). Mainly intracellular proteins, there are some hypotheses that include these proteins also on the phospholipid bilayer of the plasma membrane (Huber, Romisch et al. 1990; Heizmann 1991; Sherbet 2000). Annexins are involved with membrane fusion and secretion (Burger, Berendes et al. 1993; Ohsawa, Imai et al. 1996; Benz and Hofmann 1997; Sherbet 2000),  $\text{Ca}^{2+}$ -channel activity (Huber, Romisch et al. 1990; Heizmann 1991; Crumpton 1992), inhibition of protein kinase C (*in vitro* and *in vivo*) (Heizmann 1991; Celio 1996; Ohsawa, Imai et al. 1996), neutrophil migration (Benz and Hofmann 1997), phospholipase  $\text{A}_2$  and of blood coagulation (Smith, Kaetzel et al. 1990; Heizmann 1991; Celio 1996; Ohsawa, Imai et al. 1996; Benz and Hofmann 1997; Bandorowicz-Pikula 2003; Wang, He et al. 2006), membrane trafficking (Celio 1996; Benz and Hofmann 1997), and the formation of ion channels in the cells (Sherbet 2000).

Some particular studies (Huber, Romisch et al. 1990; Huber, Schneider et al. 1990; Huber 1992), present prominent results regarding the crystal structure of that group of proteins – in particular annexin V. They present a common fold of four domains (Celio 1996). Each domain has five helices with a short interconnecting loop (Celio 1996). There are two loops with the phospholipid-binding capacity in a  $\text{Ca}^{2+}$ -dependent manner. Those loops are located between helices A and B (domain 3) and helices D and E (domain 2) (Huber, Romisch et al. 1990; Huber, Schneider et al. 1990; Barton, Newman et al. 1991; Huber 1992; Benz and Hofmann 1997) (Figures 8 and 9).



**Figure 8:** Stereoviews of annexin V. The helical segments (A, B, C, D, and E) are shown as cylinders and the four repeats are indicated by Roman numerals. Part (a) is the view onto the convex face from the membrane. The central pore appears widely open. Part (b) is the side view. The convex face on top is the calcium- and membrane-binding site (Crumpton 1992).



**Figure 9:** Stereo ribbon plots of human annexin V viewing (top) from the molecule side and (bottom) from the convex side harboring the highly conserved calcium-binding sites. The high- and low-calcium forms are superimposed, showing the conformational change in domain III. Calcium ions are depicted as yellow spheres and the four domains are indicated in different colors: domain I, green; domain II, dark blue; domain III, light blue (high-calcium form) and pink (low-calcium form); domain IV, red. The side chain of Trp-187 is shown in ball-and-stick representation. Graphics were created with MOLSCRIPT (Liemann and Huber 1997).

The annexins present a conserved homologous helical domain (Schlaepfer and Haigler 1990; Smith, Kaetzel et al. 1990) with 70 amino acids, repeated four times (eight times in annexin VI (Smith, Kaetzel et al. 1990; Heizmann 1991; Crumpton 1992; Celio 1996; Ohsawa, Imai et al. 1996; Benz and Hofmann 1997; Liemann and Huber 1997)) in the overall structure (Figure 10). Each conserved domain is preceded by a variable region of 7 to 40 amino acids (Figure 11) (Heizmann 1991; Celio 1996). Those proteins have the capacity to bind phospholipids in a calcium-dependent manner (Heizmann 1991; DeFrancesco 1997). The calcium and phospholipids-binding sites are present in that structure (repeated domains) (Heizmann 1991; Crumpton 1992).

Cytosolic (Crumpton 1992) and water soluble proteins (Huber, Romisch et al. 1990) bind to negatively charged phospholipids (Wang, He et al. 2006) having structural and functional features of both soluble and integral membrane proteins (Huber, Romisch et al. 1990; Huber 1992). Annexins can be regulated by phospholipids and  $\text{Ca}^{2+}$  (Benz and Hofmann 1997), participating actively in the control of intracellular calcium (Smith, Kaetzel et al. 1990; Heizmann 1991).

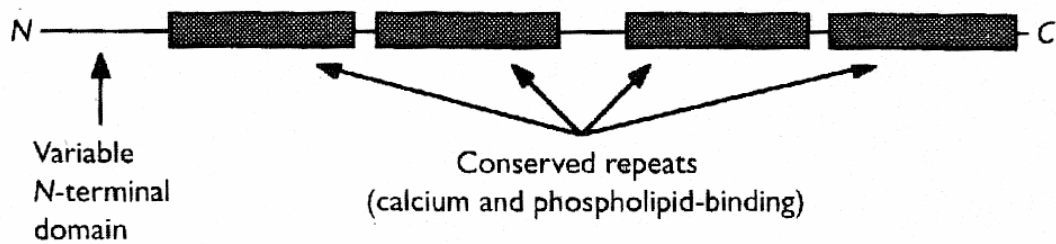
Almost all annexins present an  $\alpha$ -helical structure (Huber 1992; Benz and Hofmann 1997). Each of the four repeats of annexin V is folded into a compact domain with a similar structure (Burger, Berendes et al. 1993). The annexins present a globular structure. Annexins are water soluble with polar residues on the surface (Huber, Romisch et al. 1990) facing the hydrophilic as well as the hydrophobic phase (Huber 1992). They present an ability to bind calcium without the use of the EF-hand motif (helix-loop-helix configuration) (Smith, Kaetzel et al. 1990); (Heizmann 1991; Burger, Berendes et al. 1993; Benz and Hofmann 1997; Bandorowicz-Pikula 2003). The subfamily of calcium binding proteins (non-EF-hand CBPs) (Table 2) binds to some phospholipids in a calcium-dependent fashion (Heizmann 1990).

Generally, annexin V is present at the intracellular level, although it can be translocated to the plasma membrane (Sherbet 2000). There have been attempts to establish a relation between the cell-cycle stage and annexins' proliferation (Celio 1996). However, there are no prominent conclusions yet for this hypothesis.

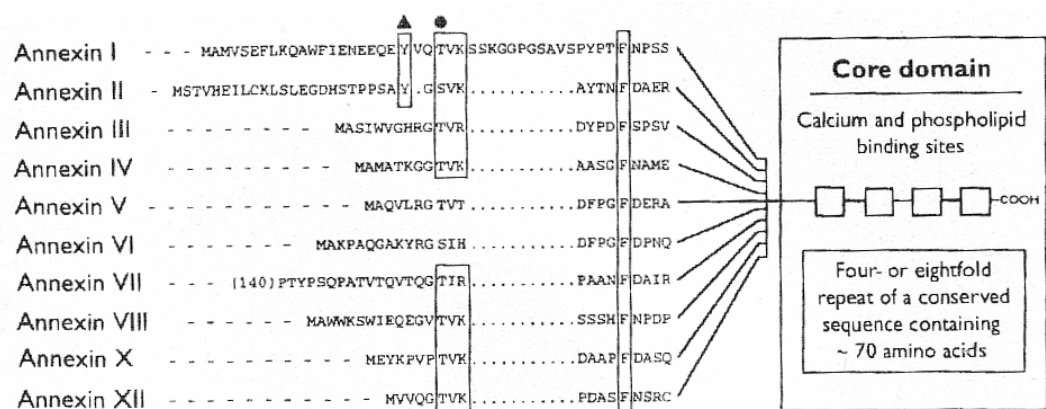


**Table 2:** Non-EF-hand calcium binding proteins (Sherbet 2000).

Non-EF-Hand CBP	Function
Annexins	Membrane-related functions; adhesion; fusion; exocytosis; possible cell-cycle-related expression; morphogenesis; differentiation.



**Figure 10:** The canonical annexin structure. A typical four-repeat annexin is shown, emphasizing the arrangement of the calcium/phospholipid binding sites (Crumpton 1992).



**Figure 11:** Annexin protein structure. The sequences of the N-terminal domains of the annexins show limited areas of sequence similarity (boxed). ▲, tyrosine kinase phosphorylation site; ●, probable protein kinase C phosphorylation site (Crumpton 1992).

### 3.1. Annexin V

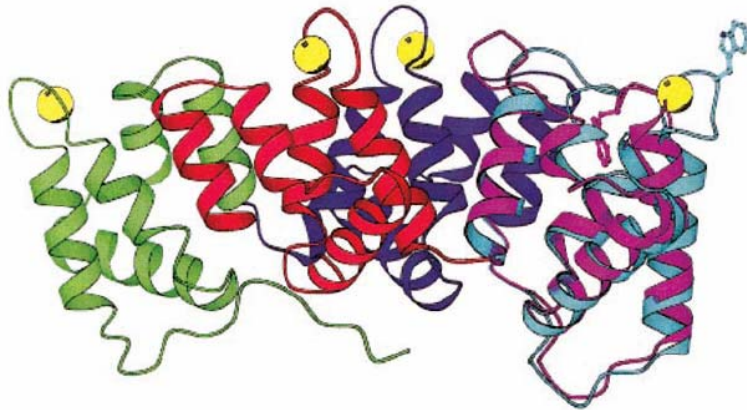
Annexin V, a monomeric protein (Seaton, Head et al. 1990), presents a structure with four homologous repeated domains. The tertiary structure of annexin V shows only one single cysteine residue hidden by that structure. It is a non-glycosylated protein, although it has potential O-glycosylation sites (without N-linked glycosylation sites) (Romisch and Paques 1991).

*In vitro* studies show that annexin V reveals an ion channel activity, binding in a calcium-dependent manner to acidic phospholipids (e.g., phosphatidylserine (Ohsawa, Imai et al. 1996; Kling 1997; Bandorowicz-Pikula 2003; Sigel and Sigel 2004)).

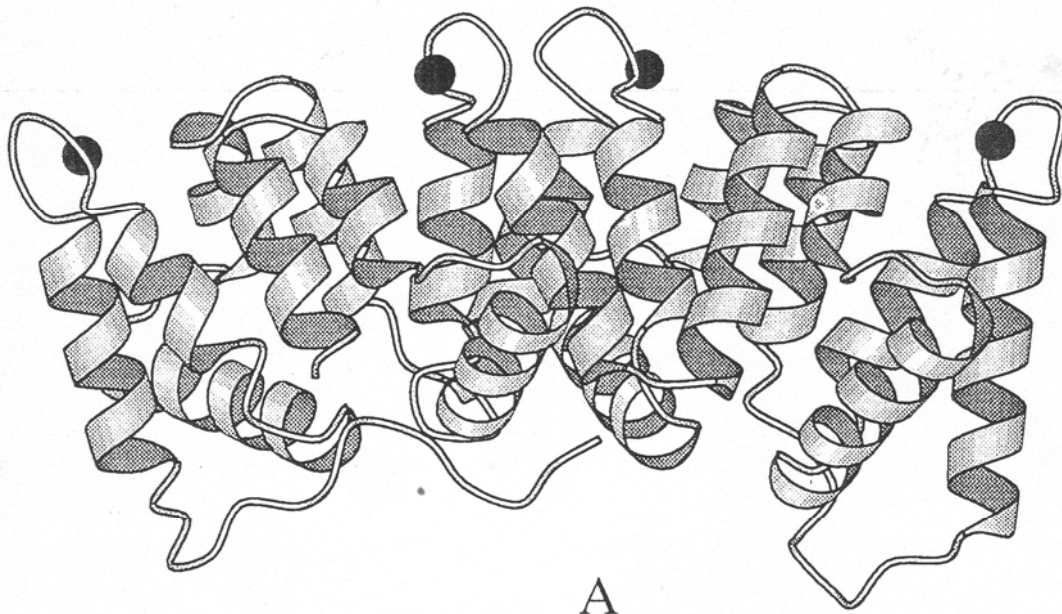
There is some evidence that three domains present  $\text{Ca}^{2+}$  binding sites (Celio 1996). The molecule presents three  $\text{Ca}^{2+}$  binding sites (Ca1 to Ca3) (Huber 1992), situated at the convex face of the molecule as possible phospholipid binding sites (Celio 1996), which have structural similarity to the calcium site present on phospholipase  $\text{A}_2$ . Huber et al., conclude that membrane-bound and soluble annexin present very similar structures, since that the membrane-anchoring region is located on the convex face (Huber 1992).

The *in vivo* role of the annexin V is still unclear. However, it has been proposed that it is involved in exocytosis (Burger, Berendes et al. 1993; Celio 1996; Bandorowicz-Pikula 2003), anti-coagulation (Burger, Berendes et al. 1993), anti-inflammation (Burger, Berendes et al. 1993), anti-thrombotic processes (Huber, Romisch et al. 1990; Benz and Hofmann 1997), and cell differentiation or replication (Burger, Berendes et al. 1993; Celio 1996).

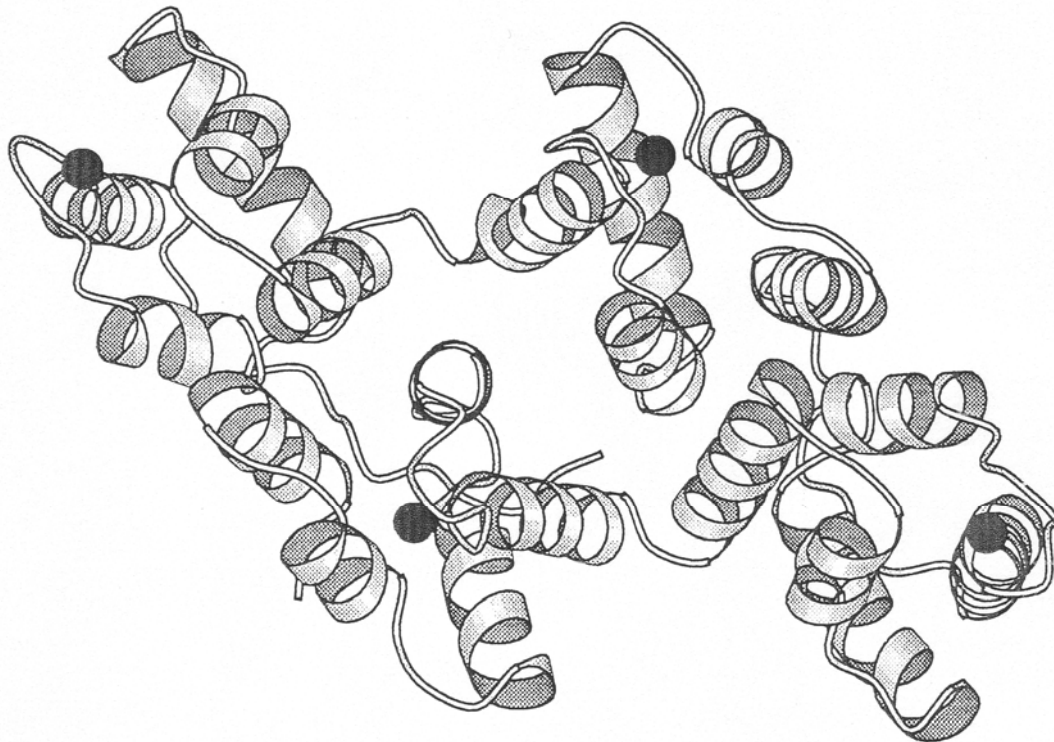
Annexin V has a single polypeptide chain that is 320 amino acids long (Appendix D) (Celio 1996; Mira, Dubois et al. 1997) (including the first methionine) with an evaluated molecular mass of 35,935 Da (Mira, Dubois et al. 1997) and dimension of 64 x 40 x 30 Å<sup>3</sup> (Huber, Romisch et al. 1990), folded into four domains of similar structure arranged in an almost planar cyclic array (Huber 1992; Benz and Hofmann 1997). Each domain is composed of five  $\alpha$ -helices with an approximately 18 Å diameter (Figures 8, 9, 12 and 13).



**Figure 12:** *Homo sapiens* annexin V crystal structure. The ribbon drawing illustrates the highly  $\alpha$ -helical folding of the protein core that forms a slightly curved disk. Different colors were chosen to highlight the four annexin repeats that are given in green (repeat I), blue (repeat II), red (repeat III), and violet/cyan (repeat IV). The NH<sub>2</sub>-terminal domain appears unstructured and extends along the concave side of the molecule (green). The high and low Ca<sup>2+</sup> forms are shown in a superposition revealing the conformational change in repeat III, which leads to an exposure of Trp-187 (violet for the low and cyan for the high Ca<sup>2+</sup> form). Bound Ca<sup>2+</sup> atoms are depicted as yellow spheres (Benz and Hofmann 1997).



A



B

**Figure 13:** (a) The structure of human annexin V (first solved by Huber *et al.* 1992) as seen from the side. The ribbons represent  $\alpha$ -helices. The concave side (on the bottom)

bears the N-terminus, whereas the  $\text{Ca}^{2+}$ -binding sites ( $\text{Ca}^{2+}$  ions are depicted as black spheres) are located on the convex side. **(b)** Structure as seen from the concave side. The four domains built up by five  $\alpha$ -helices are visible. The ion conductance pathway is in the middle. The location of the major  $\text{Ca}^{2+}$ -binding reflects the symmetry of the molecule (Celio 1996).

A hydrophobic pore is present in the center of the molecule that is associated with the calcium-selective channel (Huber 1992). Annexin V has an overall flat, slightly curved shape with a convex and concave face (Huber 1992). It can be found in a variety of eukaryotic cells, primarily located in the cytoplasm (cytosolic protein) although annexin V can be present outside the cell and in the nucleus (intra- and extracellular locations). Annexin V is thought to bind to planar PS surfaces (Rauch and Janoff 1990).

Annexin V is a member of calcium-dependent phospholipid binding proteins (Klee 1988; Ohsawa, Imai et al. 1996; Brush 2000) with a high binding affinity ( $k_d = 0.5\text{-}7\text{ nM}$  - dissociation constant) (Wang, He et al. 2006) to PS-containing phospholipid bilayer (Table 3) (Thiagarajan and Tait 1990; Thiagarajan and Tait 1991; Tait, Engelhardt et al. 1995; DeFrancesco 1997; Tait, Smith et al. 2002; Wang, He et al. 2006). The first time that annexin V was isolated, it was obtained from a placenta (placental anticoagulant protein is another possible denomination for this protein) (Bandorowicz-Pikula 2003), evidencing good blood anti-coagulant properties (Benz and Hofmann 1997; Wang, He et al. 2006). The researchers started studying programmed cell death to detect the loss of membrane asymmetry considered one of the major events directly related with apoptosis (Kling 1997; DeFrancesco 1999; Bandorowicz-Pikula 2003; Blondelle 2006).

**Table 3:** General properties of the annexin V. Adapted from (Heizmann 1991; Celio 1996).

<b>Protein</b>	<b><math>M_r \times 10^{-3}</math></b>	<b>pI</b>	<b>Phosphorylation</b>	<b><math>Ca^{2+}</math>-binding</b>
Annexin V (monomer)	33-35	4.8		4 sites



Cytoskeleton (Burger, Berendes et al. 1993; Ohsawa, Imai et al. 1996), endoplasmic reticulum, and plasma membranes are prominent sites for *in vivo* action of annexin V. There is some evidence postulating that annexin V might participate in an important interaction in the regulation of cytoskeleton elements attached to membranes (Ohsawa, Imai et al. 1996). Structural features of annexin V include channel forming membrane proteins with polar surface characteristics of soluble proteins, due to the presence of polar and charged amino acid residues present on its surface (Huber, Romisch et al. 1990). They are involved with processes of membrane fusion, exocytosis and cell signal pathways (Huber, Romisch et al. 1990; Burger, Berendes et al. 1993).

Annexin V has an interaction with different cell-membrane components (Huber, Romisch et al. 1990) that are involved in the structural organization of the cell, intracellular signaling by enzyme modulation (Ohsawa, Imai et al. 1996), and ion fluxes and growth control. Annexin V can act as atypical calcium channels (Crumpton 1992).

Some *in vivo* studies concluded that annexin V accumulates preferentially at arterial and intracardiac thrombi (Flaherty 1990; Tait, Cerqueira et al. 1994; Stratton 1995; Tait, Engelhardt et al. 1995), being characterized as a potential thrombus-targeting agent (Tait, Engelhardt et al. 1995) with anticoagulant properties (Ohsawa, Imai et al. 1996; Benz and Hofmann 1997; Brush 2000). In some research studies, annexin V was isolated from lung, heart and liver membranes. Annexin V can be used as an apoptotic cell-marker in *in vivo* studies (Wang, He et al. 2006).

There is some evidence that shows that annexin V presents two distinct binding types:  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent. When annexin V binds to phospholipids, the formation of voltage-gated calcium-selective channels with associated membranes can occur (Burger, Berendes et al. 1993).

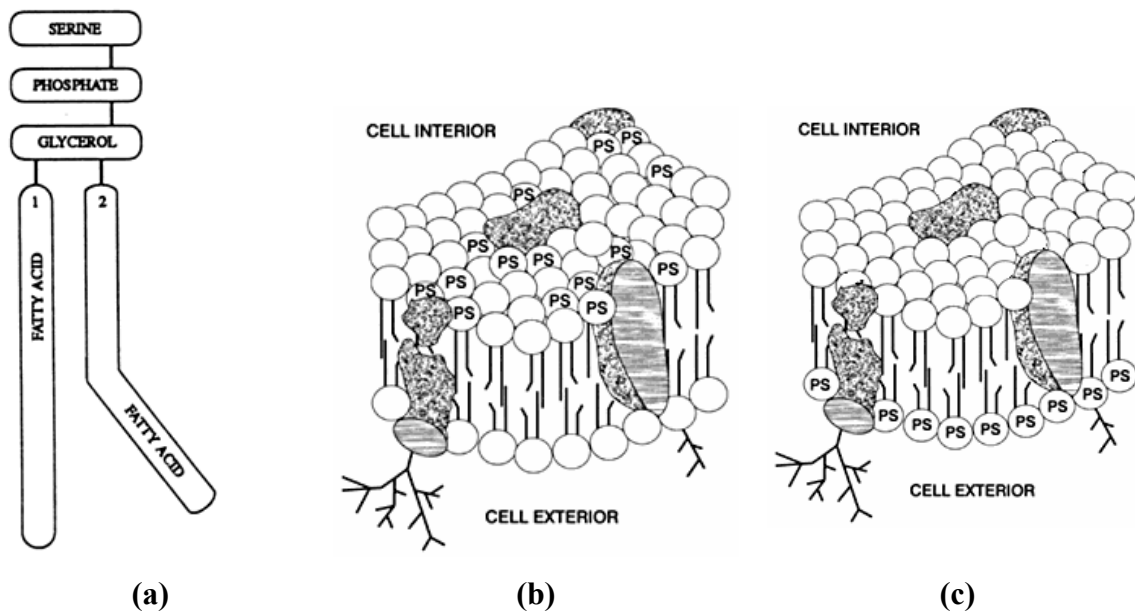
According to its intrinsic properties, annexin V can be used as an apoptotic cell marker. When combined with a radioisotope or fluorochrome (Brush 2000), it constitutes a powerful imaging system (DeFrancesco 1997; DeFrancesco 1999; Brush 2000). Annexin V constitutes the basis for a sensitive flow cytometric assay for cells undergoing apoptosis (DeFrancesco 1999; Sigel and Sigel 2004). Diseases and mechanisms, such as

heart failure, aplastic anemia, or transplant rejection, involve high apoptosis occurrence; therefore, it is possible to image them using annexin V (Sigel and Sigel 2004).

In this study, we used *E. coli* in order to express annexin V, since in that system annexin V can be very easily expressed in a soluble form with the great advantage of non-fusion with another protein (Celio 1996).

#### 4. Phosphatidylserine

Phospholipids (phosphatides) are a major structural component of the cell membrane and can be involved with some cancer signaling (Sherbet 2000). Phosphatidylserine (PS) is an anionic phospholipid that, in normal cells, is exclusively present and tightly segregated in the internal leaflet of the plasmatic membrane (Verkleij, Zwaal et al. 1973; Gordesky, Marinetti et al. 1975; Zwaal, Roelofsen et al. 1975; Marinetti and Crain 1978; Schlaepfer and Haigler 1990; Ran, Downes et al. 2002; Huang, Bennett et al. 2005), i.e., it presents an asymmetric and heterogeneous distribution (Diaz and Schroit 1996; Schönthal 2004) (Figure 14). That characteristic asymmetry (Ran, Downes et al. 2002) is maintained by a particular enzyme – aminophospholipid translocase – that transports PS to the cytoplasm (Seigneuret and Devaux 1984; Daleke and Huestis 1985; Diaz and Schroit 1996; Ran, Downes et al. 2002). When the cell is under certain conditions, the PS is transported from the cytoplasm to the external leaflet of the membrane (Brush 2000), by activation of a  $\text{Ca}^{2+}$ -dependent enzyme scramblase (responsible for the bi-directional lipids transport) or by inhibition of the aminophospholipid translocase (Seigneuret and Devaux 1984; Diaz and Schroit 1996).



**Figure 14:** (a) Molecular organization of phosphatidylserine (PS). (b) PS is preferentially distributed in the inner leaflet of the cell membrane. PS has an important role in the association with key membrane proteins (c) On endothelial cancer cells, the PS is externalized on the cell surface.

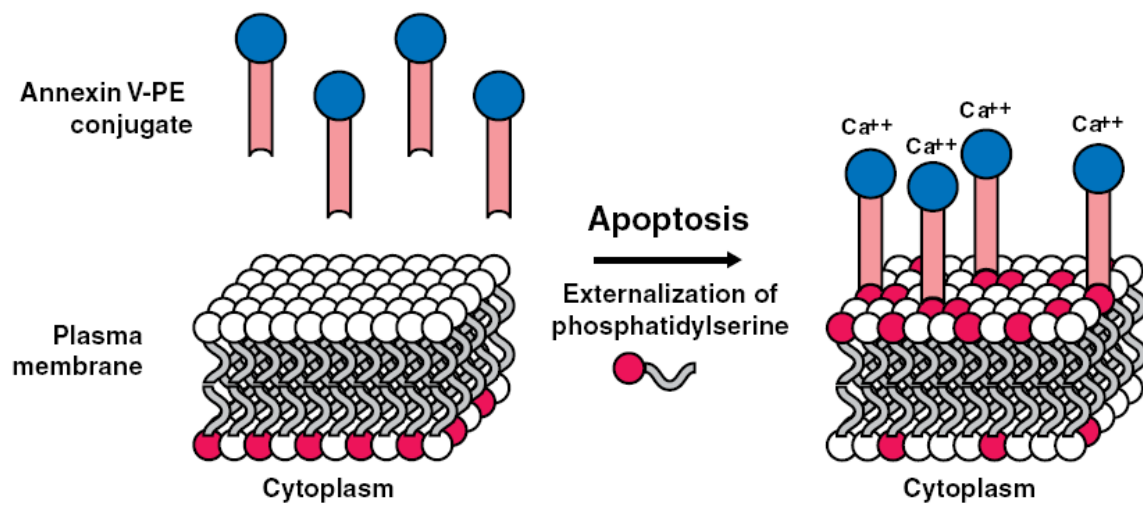
([http://www.springboard4health.com/books\\_online/ps/phosphatidylserine.html](http://www.springboard4health.com/books_online/ps/phosphatidylserine.html)).

When normal cells are exposed to some stress conditions, they undergo oxidative stress that can be caused by the presence of cancer cells. It is known that hypoxia/reoxygenation conditions and the presence of activating cytokines (Ran and Thorpe 2002), leukocytes and metabolites (Beck, Luster et al. 2006) are preponderant factors of cell stress. This specific stage induces the production of reactive oxygen species. The ensuing oxidation of membrane phospholipids and the generation of calcium fluxes (Ran, Downes et al. 2002) will also provoke the inhibition of aminophospholipid translocase and/or activation of PS exporters (Verhoven, Schlegel et al. 1995).

The exposure of the cells to reactive oxygen species can contribute to the activation of several factors that promote the occurrence of apoptosis (Cooper 2004; Blondelle 2006). Therefore, it is possible to conclude that the PS can be considered abundant, accessible (Beck, Luster et al. 2006) and a marker of apoptosis (Huang, Bennett et al. 2005; Blondelle 2006), being a facilitator of macrophage phagocytosis of apoptosis cells (DeFrancesco 1997; Brush 2000; Schönthal 2004; Beck, Luster et al. 2006). Research has proven that when the cell is exposed to necrosis factors, the PS is also exposed on the external leaflet of the plasmatic membrane (Huang, Bennett et al. 2005). PS plays an important role in aging, blood coagulation, cell-cell recognition and apoptosis (Diaz and Schroit 1996; DeFrancesco 1999; Blondelle 2006).

The evidence that vascular endothelial cells present in tumors externalize PS (Ran and Thorpe 2002; Huang, Bennett et al. 2005) is an important and basic idea to the development of this study. The notion that exclusivity, i.e. the exposure of PS, does not occur in normal cells contribute to the development of an efficient and localized therapeutic and imaging system (Ran and Thorpe 2002) that allows for precise tumor localization.

Annexin V is a  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein with high affinity for PS (Figure 15) (Kling 1997; Blondelle 2006). When coupled to a fluorochrome, this protein can be used as a sensitive probe for detecting PS exposure on the outer leaflet of the plasma membrane (Blondelle 2006).



**Figure 15:** Schematic representation of annexin V illustrating the  $\text{Ca}^{2+}$ -phospholipid binding affinity (BD Biosciences 2007).

In mammalian cells, a higher concentration of PS – an excellent marker of tumor vasculature (Ran and Thorpe 2002; Huang, Bennett et al. 2005) – is observed in the plasma membrane in comparison with the other cell's organelles, also being the most abundant lipid of the plasma membrane (Huang, Bennett et al. 2005).

The increasing platelet procoagulant response verified in certain tumors is considered one contributing factor in the translocation of PS to the external leaflet of the membrane (Bevers, Comfurius et al. 1983; Rao, Tait et al. 1992; Yuan, Yang et al. 2004). The presence of thrombin (Qu, Conroy et al. 1996; Ran, Downes et al. 2002; Huang, Bennett et al. 2005), viral infection (Vangeelen, Slobbevandrunen et al. 1995), hyperlipidemia (Lupu, Moldovan et al. 1993), inflammatory cytokines (Huang, Bennett et al. 2005) and hydrogen peroxide (Ran, Downes et al. 2002) contacting endothelial cells promotes PS externalization. Cell degranulation (Demo, Masuda et al. 1999), cell aging (Herrmann and Devaux 1990), malignant transformation, cell activation (Ran, Downes et al. 2002; Huang, Bennett et al. 2005), cell migration (Vogt, Ng et al. 1996), cell injury (Ran, Downes et al. 2002), programmed cell death (Bombeli, Karsan et al. 1997; Kling 1997; Blankenberg, Katsikis et al. 1998; Blondelle 2006), intracellular fusion of trophoblasts (Adler, Ng et al. 1995) and myoblasts (Sessions and Horwitz 1981) and activation of platelets (Benz and Hofmann 1997; Yuan, Yang et al. 2004) are other factors associated with the induction of PS externalization. These stimuli when combined may amplify the PS externalization (Ran, Downes et al. 2002).

Regarding the presented information above, there are several agents responsible for the PS externalization. However, in some cases, spontaneous appearance of PS in malignant cells in the absence of cell injury or exogenous activators was reported (Utsugi, Schroit et al. 1991).

There are several characteristics pertaining to PS in cells: they are abundant (PS is present at more than  $10^6$  molecules per cell); they are on the luminal surface of tumor endothelium, which can be directly accessible for binding by vascular targeting agents in the blood; they are present in a significant percentage of tumor endothelial cells in diverse solid tumors; and they appear to be absent from endothelium in all of the normal tissues (Ran, Downes et al. 2002).

*In vivo* studies have shown that the exposure of PS occurs on tumor endothelial cells in various solid tumors, including metastatic tumors and drug-resistant tumors, and are on the luminal side of tumor endothelium, which is freely accessible for binding by targeted drugs (Sanchez 2004; Bianco, Kostarelos et al. 2005).

A detailed analysis (McDonald and Baluk 2002) has shown that tumor vessels present structure deficiencies showing thin, leaky and tortuous walls, irregular shape, dilatation (Sanchez 2004), insufficient supportive pericytes and can have dead ends (Sanchez 2004). This anomaly increases the sensitivity to radiotherapy, vascular targeting agents (VTA) and chemotherapeutic drugs (Sanchez 2004). Another important aspect is that endothelial cells present in normal vasculature are quiescent and nondividing, whereas tumor vascular endothelial cells are active and proliferative (Cooper 2004).

Because of annexin V's high affinity and specificity for phospholipids (particularly PS) (Huang, Bennett et al. 2005), it can be used for targeting or the imaging of tumor blood vessels (target molecule) (Blondelle 2006). More sophisticated systems include the possibility of annexin V being used to deliver a radionuclide, a cytotoxic drug (Sanchez 2004) or coagulant to tumor vessels (Ran, Downes et al. 2002).

In some *in vivo* studies, annexin V was found in cancer cells and in its vascular endothelium and around necrosis regions (Ran, Downes et al. 2002). That affinity property permitted the use of annexin V to image activated platelets (Tait, Engelhardt et al. 1995) in thrombi apoptotic cells in cardiac allografts undergoing rejection, cyclophosphamide-treated lymphomas, and anti-Fas antibody-treated livers in rodents (Shaughnessy, Buchanan et al. 1989). The stress conditions present in the tumor microenvironment provoke the PS exposure at the tumor vasculature level (Ran, Downes et al. 2002; Huang, Bennett et al. 2005).

The activation of tumor endothelium is caused mainly by tumor-derived interleukin-1 and tumor necrosis factors, which activate the endothelium and induce expression of cell adhesion molecules, (Shaughnessy, Buchanan et al. 1989; Orr, Wang et al. 2000), reactive oxygen species (ROS) generated by leukocytes that adhere to the endothelium (Orr, Wang et al. 2000), ROS generated by tumor cells themselves as a byproduct of metabolism (Shaughnessy, Buchanan et al. 1989; Soares, Shaughnessy et al.

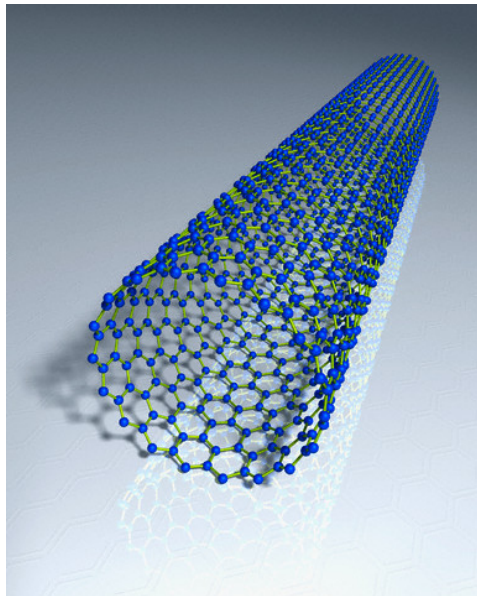


1994) or as a result of exposure to hypoxia followed by reoxygenation (Zulueta, Yu et al. 1995).

#### 5. Single walled carbon nanotubes (SWNTs)

In order for the annexin V-PS *in vivo* binding be possible, it is necessary to use an efficient carrier that, when injected in the bloodstream, should not interfere with the internal medium.

The development of new nanotechnology makes possible the synthesis of nanostructures called nanotubes. These structures can have multiple walls (MWNT) or a single wall (SWNT) (Figure 16).



**Figure 16:** General structure of a SWNT. A single-walled carbon nanotube (SWNT) is made up of benzene rings (hexagonal carbon units) and rolled into a tube (<http://data.engin.umich.edu/umseds/kc135/nanotubes/report/FinalReport.htm>).

In this study, a single walled carbon nanotube suspension was obtained by sonicating a CoMoCAT sample using as dispersant sodium cholate (NaC, from Sigma, St. Louis, MO) or low-viscosity sodium carboxymethylcellulose (CMC, from Sigma, St. Louis, MO).

The unique size, shape and conformation structure are physical properties that make SWNTs a potential application to biological/medical studies (Liu, Cai et al. 2007). The development of new applications using SWNTs constitutes a promising field. Nanomolecular transporters for drug delivery is one example of some possible SWNT applications for potential new therapies (Liu and Wang 2007).

SWNTs have the ability to cross the cell membranes (endocytosis) to deliver proteins and nucleic acids (Liu, Cai et al. 2007). Because of their high hydrophilicity, water-solubilized nanotubes do not present toxicity, even at high concentrations. *In vivo* biodistribution and tumor targeting studies, using animals, showed that there is a specific elimination profile for SWNTs – being eliminated in the urine with a small accumulation at the liver and reticuloendothelial system (RES) (Liu, Cai et al. 2007).

Carbon nanotubes present a very defined structure, showing a cylindrical structure with dimensions that can vary between several hundred nanometers to several micrometers (length) and 0.4-2 nm (diameter) for SWNTs (Lin, Taylor et al. 2004). In the present study, (6,5) and (7,6) nanotubes were used with diameter of 0.757 and 0.895 nm, respectively (Bachilo, Balzano et al. 2003). Carbon nanotubes have several optical, mechanical, electronic and thermal properties of interest (Cherukuri, Gannon et al. 2006; Liu and Wang 2007) such as high electrical conductivity, high aspect ratio (length is approximately equal to 1000 times the diameter), low thermal expansion coefficient, high thermal conductivity, very high tensile strength, high elasticity (approximately 18% to failure), good electron field emitters and high flexibility (can be bent considerably without damage) (<http://www.azonano.com/details.asp?ArticleID=980>; <http://www.cheaptubesinc.com/applications.htm>). The high aspect ratio is an important property, since it is possible to incorporate several types of molecules with different sizes from small molecules to proteins (Kohli and Martin 2005).

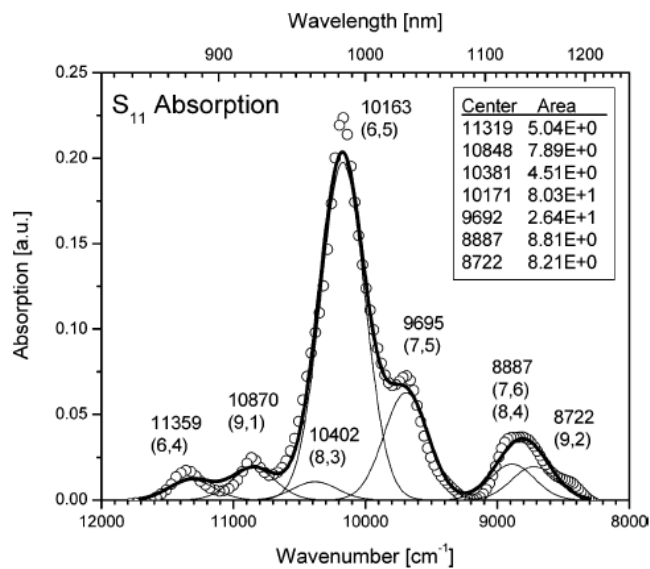
Those properties of the nanotubes constitute a promising field with potential technological applications (Fu 2002). SWNTs are “rolled-up” artificial nanostructures (Cherukuri, Gannon et al. 2006) of a unique layer of graphene (Lin, Taylor et al. 2004; Cherukuri, Gannon et al. 2006). Carbon nanotubes are cylindrical carbon allotrope molecules that can be used in several different fields of study (e.g., nano-electronics, optics, materials applications, biological and biomedical systems (Lin, Taylor et al. 2004) and biosensor and bioreactor systems).

Biochemical sensors, diagnostic imaging contrast agents, targeted chemotherapeutics, *in vitro* cell markers and photoablative therapy agents (Cherukuri, Gannon et al. 2006) are examples of SWNT biomedical applications. The use of nanotubes in biotechnology is possible since specific molecules, such as enzymes or proteins, can be immobilized on the surface or in the hollow cavity of nanotubes (Lin, Taylor et al. 2004; Liu and Wang 2007). The fact that nanotubes present two distinct surfaces (inner and outer surface) shows they can be functionalized, presenting different properties from the inner surface to the outer surface (Kohli and Martin 2005; Liu and Wang 2007). Nanotube-protein conjugates can afterward be integrated into biological systems. Generally, because of its properties, nanotubes constitute an excellent mean of transportation *in vivo*, allowing the delivery of a specific compound (e.g., drugs, peptides, nucleic acids (Liu and Wang 2007)) into a concrete region, including the possibility of performing gene and protein therapy (Fu 2002). The delivery of genetic material into living cells – transfection (Kohli and Martin 2005) – is another example of a nanotube application. Antigen recognition, DNA hybridization, enzyme-catalyzed reaction (Lin, Taylor et al. 2004), use as vehicle for enzyme encapsulation, DNA transfection, biosensors and drug delivery (Kohli and Martin 2005; Liu and Wang 2007) are some examples of how nanotubes can be used.

Different types of nanotube functionalization are available, it being possible to adjust the nanotubes to a specific desired property. For example, the functionalization with PEG (PEGylation) allows nanotube solubilization, increasing the biocompatibility (Kohli and Martin 2005; Liu and Wang 2007; Liu, Cai et al. 2007) that is an important factor to perform some biological studies (Fu 2002; Lin, Taylor et al. 2004). Generally,

the nanotube suspension is required to be highly water-soluble, presenting a dark-colored aqueous suspension (Huang 2002). High bioavailability, biocompatibility, nontoxicity (Kam, O'Connell et al. 2005) and target ability are important properties present in functionalized nanotubes.

It is known that biological systems present a high transparency in the near-infrared (NIR) light window (700-1100 nm) (Kam, O'Connell et al. 2005; Liu and Wang 2007). The characteristic strong optical absorbance of SWNTs in this wavelength range – a property related with the electronic band structures of nanotubes – can be evaluated by analysis of a  $S_{11}$  absorption spectrum. The spectrum shows the presence of a characteristic peak at 980 nm (Lolli, Zhang et al. 2006) (Figure 17).



**Figure 17:** Fitting of the S<sub>11</sub> absorption spectrum for SWNT sample synthesized on CoMo/SiO<sub>2</sub> at 750 °C. Constrained fwhh =  $328.5 \pm 1.1$ ,  $R^2 = 0.989$  (Lolli, Zhang et al. 2006).

The strong optical absorbance makes it possible to perform optical stimulation of the nanotubes, when injected into the organism (Kam, O'Connell et al. 2005). When, the SWNTs are bound to specific receptors *in vivo*, the continuous irradiation with NIR light on a specific and determined region can promote cell death because of a local temperature increase (Figure 18) (Kam, O'Connell et al. 2005; Liu and Wang 2007). However, the cells not expressing those receptors will not be injured.

Because the nanotubes absorb NIR energy, the irradiation with NIR of a specific region causes a localized temperature increase. Consequently, this temperature increase contributes to the cancer cells elimination (destruction) by two mechanisms: (1) killing the tumor's endothelial cells, which leads to clotting of the blood in the tumor and cutting off the tumor's blood supply, and (2) killing the cancer cells directly by heating.

Generally, the nanotubes produced are typically interassociated, forming bundles showing an extremely low solubility in the presence of organic solvents or water (Buffa, Hu et al. 2005). In order to solve this particular problem (dispersion and solubility of single walled carbon nanotubes), different methods have been proposed such as high-shear-flow mixing, milling and ultrasonication (Buffa, Hu et al. 2005) combined with the use of dispersing agents and surfactants (Buffa, Hu et al. 2005).

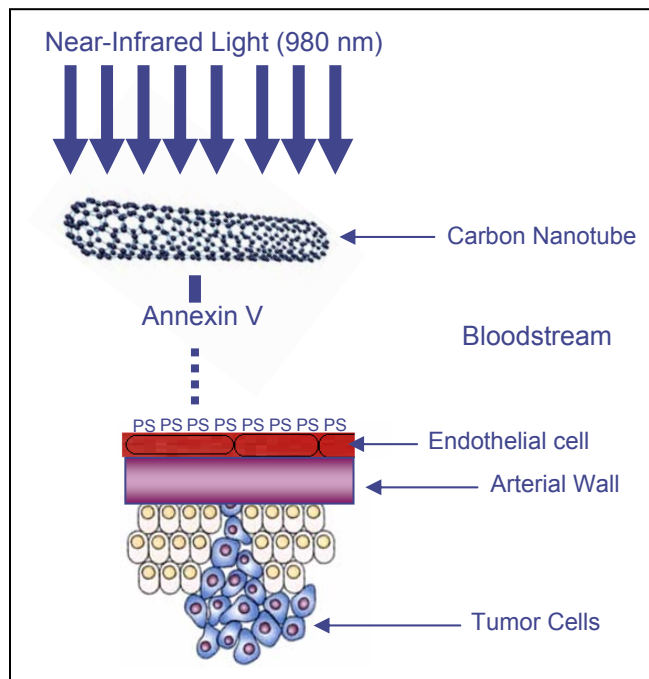
Chemical functionalization is another method that demonstrates a high potential of use. This method can involve covalent or noncovalent bindings. The high reactivity of the carbon atoms present at the ends of the nanotube structure allows the performing of several reactions that seek the establishment of a covalent-binding type. However, this method is not reliable since just a small fraction of the carbon atoms in the nanotube structure becomes functionalized.

Side-wall covalent functionalization is another available covalent attachment method that provides a high carbon atom functionality. This method gives alterations in the optical properties of the nanotubes (loss of optical absorption and photoluminescence) caused by changes induced in the graphene original structure, constituting a severe disadvantage.

Covalent attachment (Buffa, Hu et al. 2005), adsorption, and adsorption of an inert macromolecule followed by covalent attachment to the macromolecule are possible

methods to form a protein-SWNT complex (Buffa, Hu et al. 2005). Covalent attachment using the diazonium salt method allows the functionalization of SWNTs (on the sidewalls) by covalent binding of 4-hydroxymethylaniline. However, the characteristic optical absorption bands on pristine SWNTs are completely lost after functionalization.





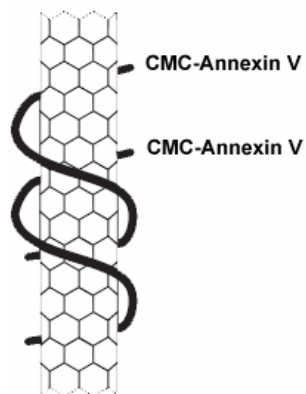
**Figure 18:** Schematic diagram showing the global mechanism of this study.

The adsorption of biomolecules to SWNTs does not induce any significant change in the intrinsic properties of the SWNTs, with the UV-Vis-NIR absorption of the SWNT's property conserved (Kam, O'Connell et al. 2005).

The use of some dispersant agents and surfactants (Buffa, Hu et al. 2005) allows for a good distribution and individually nanotube separation from each other. The use of a low concentration of sodium cholate (Graff, Swanson et al. 2005) or low-viscosity sodium carboxymethylcellulose (Figure 19) (Lolli, Zhang et al. 2006) as dispersants is encouraged because they are considered good nanotube dispersants.

Graff et al. showed that the use of sodium cholate as a dispersant allows for the formation of a stable solution-phase complex with the SWNTs (Graff, Swanson et al. 2005). Aqueous sonication (Matsuura K 2006) and organic solvent displacement (Karajanagi 2004) are examples of other available dispersant methods, although these present some problems related to the dispersion capacity (low dispersion ability) and the protein activity or structure (partial or total loss of activity).

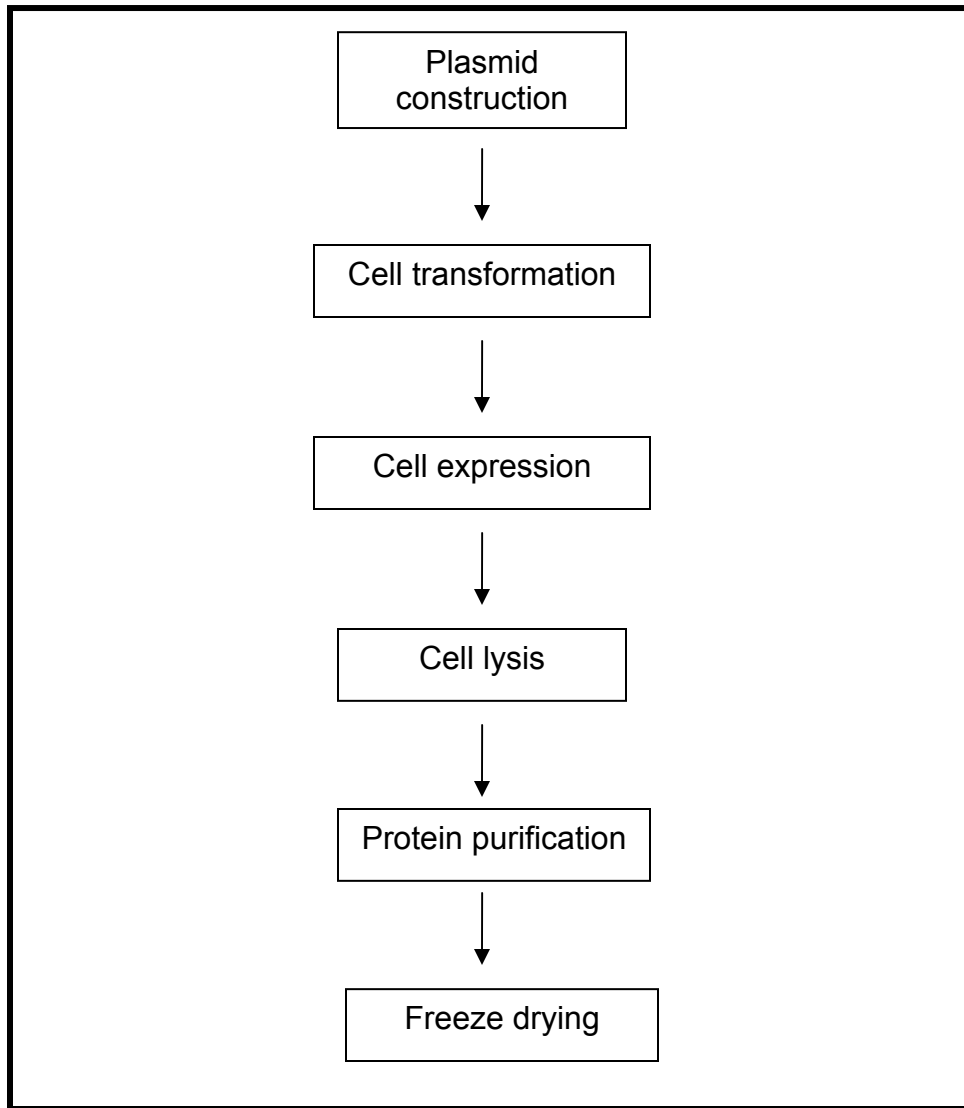
Graff et al. used the protein concanavalin-A which was adsorbed obtaining a stable solution-phase complex with the SWNTs. The protein activity and the absorption spectra were not measured and obtained, respectively. The aqueous sonication method was evaluated by Matsuura et al., determining the adsorption of four different proteins (egg white lysozyme, human serum albumin, papain and pepsin). Dispersion was observed for the human serum albumin and lysozyme, however, the dispersion was not successful for the papain and pepsin proteins. There was a significant change in the circular dichroism (CD) spectra for both lysozyme and albumin adsorbed on SWNTs compared to the CD spectra for the native proteins, indicating that the adsorbed protein was partially unfolded. Karajanagi et al. adsorbed  $\alpha$ -chymotrypsin and soybean peroxidase on the SWNTs. For soybean peroxidase, up to a maximum of only 28% of the original specific enzyme activity was retained, and the maximum loading was 575  $\mu\text{g}$  protein/mg SWNTs (= 12.0 nmol protein/mg SWNTs). For  $\alpha$ -chymotrypsin, no greater than 1% of the native specific enzyme activity was retained, and the maximum loading was 673  $\mu\text{g}$  protein/mg SWNTs (= 26.9 nmol protein/mg SWNTs). In summary, these methods either change the protein structure or result in a large loss of enzymatic activity.



**Figure 19:** A single walled carbon nanotube wrapped with CMC-annexin V. Adapted from (Liu and Wang 2007).

## 6. Synthesis of annexin V

Annexin V production requires the execution of several associated steps – plasmid construction, cell transformation into NovaBlue competent cells followed by cell expression using an appropriate inducer agent (e.g. IPTG), protein purification, and freeze drying that allows the protein storage at - 20 °C (Figure 20).



**Figure 20:** Annexin V production general overview.

## 6.1. Recombinant DNA technology

Several recombinant DNA technology techniques and strategies have been developed since the seventies, evidencing a large impact on almost all biology research fields (Glick 1998; Russell 1998; Fernandez 1999; Sambrook 2001). That research increased the interest on those areas, contributing to the development of new and promising applications that actually are being used with an increasing impact in our society (Sanchez 2004).

Nowadays, recombinant protein production is a very well studied method, allowing the production of large amounts of a desired protein. There are several factors that should be considered in order to determine the best expression system to use in a specific study. Eukaryotic and bacterial expression systems are available (Fernandez 1999). However, the bacterial expression systems are the most used by the research community. The ease of growing and lysing those cells contributes to the characterization of a powerful secretion system, where recombinant proteins can be isolated from the supernatant with high efficiency. A prokaryotic expression system is simple to handle, cost effective and allows the production of large amounts of heterologous proteins (Fernandez 1999).

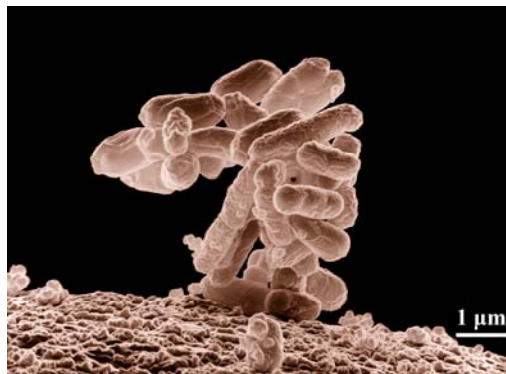
A bacterial expression system (Fernandez 1999) requires three main components: a promoter, an affinity tag and a host strain. The promoter is considered one of the most important components present in an expression vector, since it controls the genetic expression levels (Rodriguez 1987; Glick 1998). Those levels should be carefully controlled and monitored concerning the protein aggregates problem. Some studies have shown that high expression levels lead to the formation of protein aggregates, known as inclusion bodies (Asenjo 1996; Fernandez 1999). Isopropyl-beta-D-thiogalactopyronoside (IPTG) is the reagent responsible for induction of the bacterial *lacI* promoter (Fernandez 1999).

The affinity tag is very important in these systems, allowing the elution of the protein of interest. Basically, this tag binds to the affinity chromatography column, making possible the protein elution. It is important to note that the short peptide tag (His-

tag) does not induce any alteration in the recombinant protein structure – it is simply attached to the protein. The tags can easily be detached from the protein, due to the presence of a protease cleavage site placed between the tag and the heterologous protein.

To perform a cell expression protocol, it is necessary to choose an appropriate host strain. Nowadays, there are several different types of host strain systems (Asenjo 1996). Baculovirus, yeast, mammalian cells and bacteria are examples of those host strains (Asenjo 1996; Glick 1998; Russell 1998; Fernandez 1999). However, bacteria host strains are the most used system, according to its high yield relative to the other systems (Glick 1998).

In the present study, the bacteria strain chosen was *E. coli* (Figure 21), due to its good features such as, easy manipulation and quick and economic growing (Asenjo 1996; Glick 1998). Countless research groups have used *E. coli* to produce large quantities of proteins from genes with different origins (Asenjo 1996; Glick 1998). *E. coli* is an appropriate system when the soluble fractions of the protein can be expressed. It makes it possible to obtain high quantities of protein, with a correct and active structure (Asenjo 1996; Glick 1998). However, when proteins require post-translational modifications (Asenjo 1996), like glycosylation or phosphorylation, an eukaryotic expression system is used (Asenjo 1996; Fernandez 1999). The basic strategy to the protein expression is the gene cloning and its transformation on the host. Using *E. coli* it is possible to grow it in fermentation systems with high capacity for protein production (Asenjo 1996). Generally, the expression vector is a plasmid, although it is possible to use expression phages. Expression vectors in bacteria have a replication origin and selection genetic sites (Rodriguez 1987).



**Figure 21:** Low-temperature electron micrograph of a cluster of *E. coli* bacteria, magnified 10,000 times. Each individual bacterium is oblong shaped (<http://www.wikipedia.org/>).



It is important to note that there are regulated promoters that permit a large production of mRNA, when induced. Protein synthesis requires the existence of joining-sequences to the ribosome on the mRNA. These small sequences are close to the methionine-initiation codon. These sequences are present in the expression vectors, between the promoter and the cloning site. There are several regulated promoters that can be used in these systems. An interesting system uses a promoter that is recognized by the RNA polymerase T7 phage (Glick 1998). This polymerase is very selective relative to fago specific promoters. The gene of interest to express is cloned according to the promoter control.

T7 RNA polymerase is responsible for the induction of the gene transcription and translation (Glick 1998). This polymerase coding gene is controlled by an inductive promoter, like the *lac* promoter. Therefore, inducing the *lac* system (with IPTG) makes it possible to obtain the required protein. In some situations, the T7 polymerase transcription is more efficient than the bacteria polymerase transcription (Glick 1998). Usually it uses a direct expression. The protein coding sequence is connected directly to some sequences that control the transcription and translation, being preceded by a methionine initiation codon. This permits expression of cytoplasmic and secretion proteins in the bacteria cytoplasm. In the secretion proteins, the coding sequences of the secretion signal are removed and replaced by the initiation codon.

Frequently, the protein overexpression in the cytoplasm induces the formation of insoluble aggregates of protein, known as inclusion bodies. The inclusion bodies can be easily purified, and they are resistant to the protease degradation (Asenjo 1996; Fernandez 1999). However, the inclusion bodies can be considered a problem, when it is important to obtain active and soluble proteins. It is not guaranteed that the denaturation processes of the inclusion bodies will work (Fernandez 1999). The protein denaturation induces alterations in all proteins being characterized by their unfolding (Asenjo 1996).

There are several possibilities to try to avoid the inclusion bodies formation, such as temperature changes or protein expression decreasing. Sometimes, another possibility is protein secretion. The secretion using an *E. coli* host is mediated by a N-terminal signal sequence, cleaved after the protein translocation from the cytoplasm to the periplasm in

the cell, through the internal membrane. In both situations (cytoplasmic or periplasm expression) it is necessary to separate the important proteins from the bacteria proteins. However this can be a difficult process.

To produce proteins in expression systems, fusion proteins (Fernandez 1999) are now used, since these genetically engineered proteins containing one or more heterologous elements can improve the stability, solubility and purification in the solution (Glick 1998). In these particular situations, the codifying protein sequences are linked to the genes of other proteins that have the appropriate characteristics (carrier proteins). One example is the Nus A fusion protein system, in which the *E. coli* Nus A protein is linked to the N terminus of the normally insoluble protein in order to improve protein solubility (Davis 1999). Another viable alternative is protein expression using small tags (Glick 1998). These tags are added to the N or C-terminal and allow easy protein detection and/or purification (Table 4) (Glick 1998).

**Table 4:** Fusion tags available for pET constructs (Novagen 1994).

<b>Tag</b>	<b>N/C Terminal</b>	<b>Size (aa)</b>	<b>Basis for detection and/or purification</b>	<b>Applications</b>	<b>pET vector series</b>
<b>T7-Tag™</b>	N	11 or 260	monoclonal antibody	Western blot immunoprecipitation Immunofluorescence	3, 3x, 5, 9, 11, 17, 17x, 21, 23, 24, 28, pTOPE™, λEXlox®
<b>S-Tag™</b>	N	15	S-protein (103aa)	Western blot quantitative assay purification	29, 30
<b>His-Tag™</b>	N or C	6 or 10	metal chelation chromatography	His-Bind® resin purification (native or denaturing)	14, 15, 16, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30
<b>HSV-Tag™</b>	C	11	monoclonal antibody	Western blot immunoprecipitation Immunofluorescence	25, 27
<b>pelB/ompT</b>	N	20/22	potential periplasmic localization	protein export/folding	12, 20, 22, 25, 26, 27

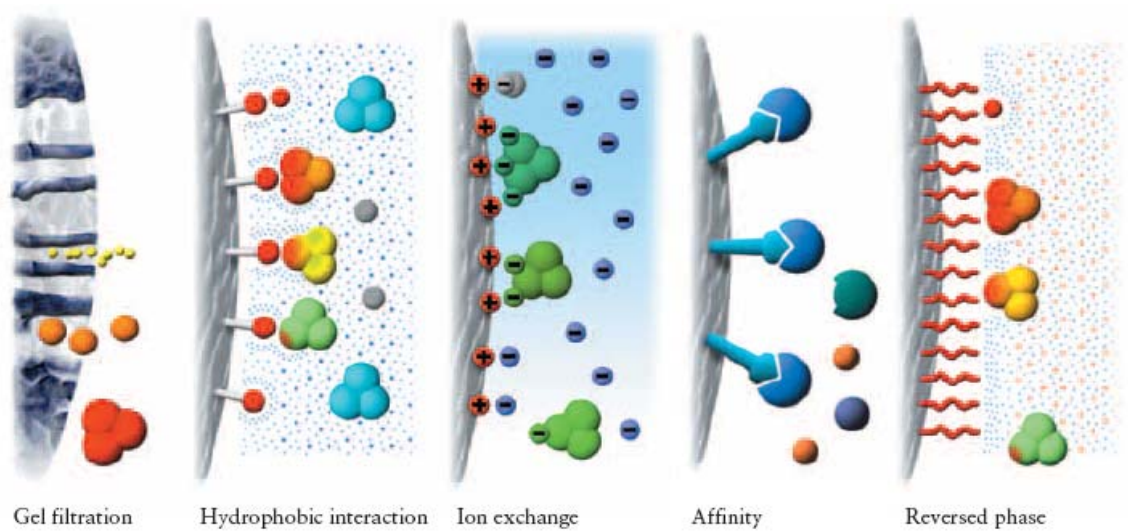
A common example of a small tag is the His-tag, which contains 6-10 histidine consecutive residues that allows protein purification by affinity chromatography (Scopes 1982; Dechow 1989). In both situations (protein fusion or affinity tags) of the protein expression, it is common to introduce a codifying sequence in the vector. This small sequence is placed between the coding relevant protein sequences and the coding carrier or tags protein sequences. These sequence codes are an amino acid sequence that can be cleaved by chemical processes or by using a protease. These methods allow the separation of the relevant protein from the affinity tag, after the purification step. Affinity and other types of chromatography used in purification are illustrated schematically in Figure 22.

The most popular bacteria used in genetic engineering studies are *E. coli* (*Escherichia coli*). According to its excellent properties (rapid and easy cultivation), this organism has been exhaustively studied. *E. coli* is a bacteria that generally is present in the animal and human intestines. The bacterial chromosome contains only DNA. However, in most eukaryotic cells, the chromosome has protein combined with DNA. Also, there is only a single chromosome in bacteria, which allows genetic expression without the interaction of a second chromosome.

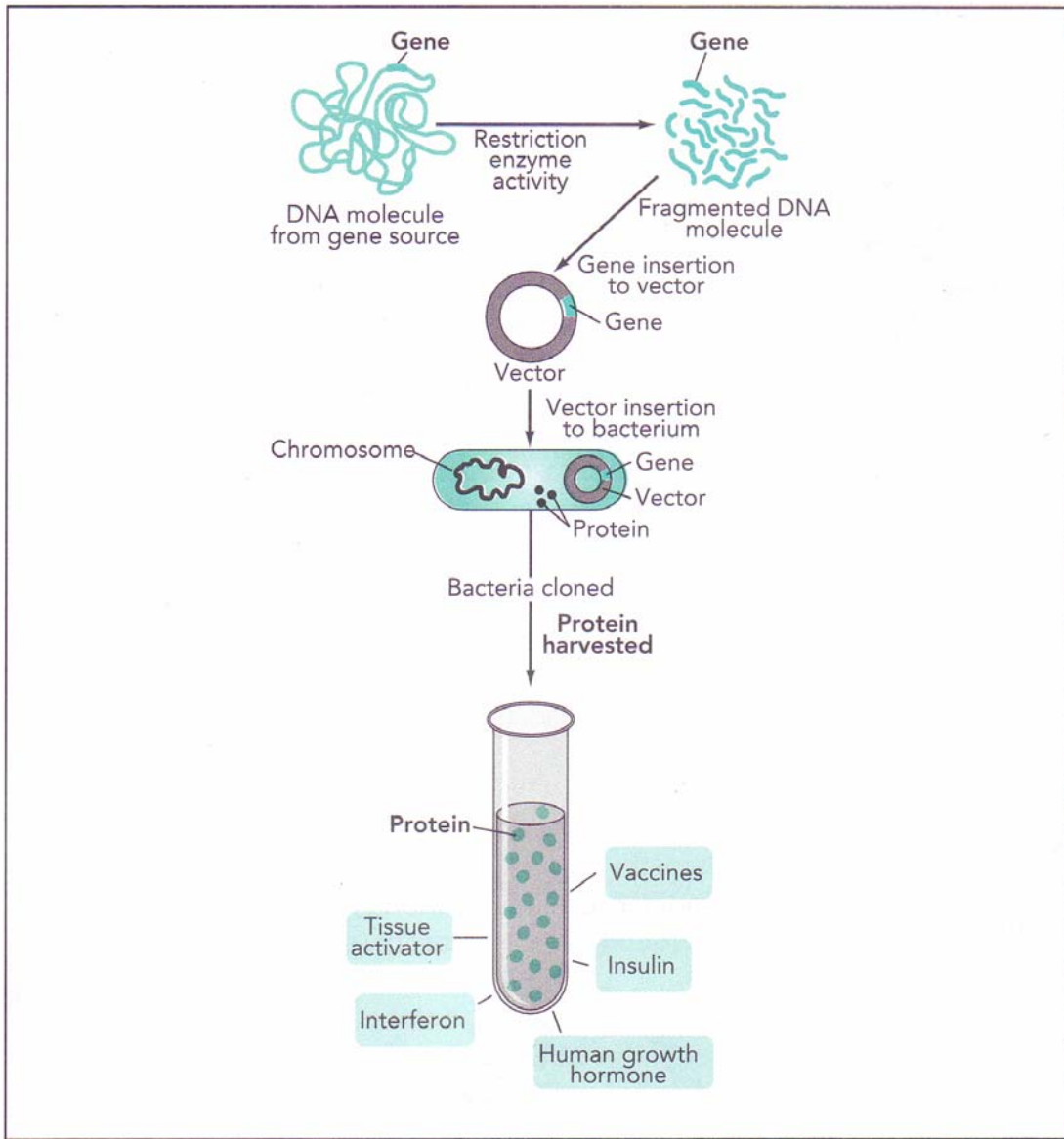
Another advantage related to the bacterial chromosome utilization is that it flows freely in the cytoplasm and is easy to reach. Foreign DNA can be easily expressed in the bacteria exactly like the bacterial DNA.

## 6.2. Overview of protein expression

Expression systems are based on the insertion of a gene into a host cell to provide its translation and expression into a specific protein (Figure 23).



**Figure 22:** Separation principles in chromatography purification (Amersham Biosciences 2002).



**Figure 23:** A review of DNA technology and genetic engineering showing the two major objectives of the process: the cloning of human genes and the large-scale production of proteins encoded by the genes. Adapted from (Alcamo 2001).

### 6.2.1. Plasmid construction

Plasmids are commonly used because they are self-replicating extrachromosomal DNA molecules. An important aspect related to plasmids is that they carry genes allowing several features, such as resistance to antibiotics, production of restriction enzymes, toxins and rare amino acids, resistance to heavy metals, catabolism of complicated organic molecules, sensitivity to mutagens and bacteriophages, resistance to bacteriophages (Fernandez 1999), determination of virulence, ability to transfer DNA across kingdoms, ability to form symbiotic relationships and others (Figure 23) (Glick 1998). It is important to mention that plasmid replication and the bacterial cell cycle can be synchronized allowing for the proliferation of hundreds of plasmid copies at each cell (Glick 1998).

The use of plasmids, also called vectors started in the 1970s and have been used in propagation, manipulation and delivery of specific DNA sequences (Glick 1998). A cloning site, a replicator and a selectable marker are three common features present in all plasmid vectors (Rodriguez 1987; Glick 1998).

The first step of the annexin V process requires the insertion of the DNA coding-region of the annexin V protein into a plasmid. Typically a plasmid is small and constituted by circular pieces of DNA, which are presented in *E. coli* (Figure 23).

In our study, a specific vector kit – pET-30 Ek/LIC vector kit (Novagen) – was used. This vector kit is characterized by a ligation-independent cloning (LIC) that is basically a directional cloning of PCR products without binding reactions or restriction enzymes (Table 5).

The ligation-independent cloning (LIC) kits (Novagen) are very useful, since they introduce a different and sophisticated approach, providing for directional cloning and eliminating the ligation step. The exonuclease stops when the 3' to 5' prime exonuclease activity of the polymerase digests the vector until reaches the nucleotide included in the reaction. This result in the vector having unique and noncomplementary overhangs. The utilization of primers with the appropriate 5' extension permits the elaboration of PCR fragments with compatible ends. Because of the fact that the ends are not complementary,

the probability of vector reannealing is very low and the resulting transformation efficiency is high. The transformation of the annealed LIC vector and insertion can occur directly into *E. coli* competent cells. This process takes advantage of the fact that cellular mechanisms can assure the establishment of a covalent bond between vector and insert sites.

The LIC vectors present an enterokinase (Ek/LIC) cleavage site adjacent to the insert site that makes possible the vector-encoded fusion removing from expressed proteins. The pET-LIC vectors are used for cloning and high level expression of cloned sequences in *E. coli*, using fusions and tags that provide an efficient detection and purification of target proteins (e.g., His-Tag<sup>®</sup>). The pET system is considered the most powerful system yet developed for cloning and expression of recombinant proteins using *E. coli* as a host strain.

The bacteriophage T7 transcription and translation signals control the cloning of target genes in pET plasmids (Fernandez 1999). A source of T7 RNA polymerase present in *E. coli* promotes the expression. When totally induced, a high percent of the cell's resources is used to target gene expression. The expected product can be higher than 50% of the total cell protein a few hours after induction with IPTG. The expression levels can easily be controlled and monitored by carefully adjusting the inducer agent (IPTG). This system should be carefully optimized in order to maximize the protein production yield (Asenjo 1996; Fernandez 1999). Another advantage of this system is related to the fact that the target genes are not transcribed during the pre-induction stage (Fernandez 1999; Novagen 2006).

The pET vector DNA, the induction control clone and the host bacterial strains BL21(DE3) are included in the pET expression kit (Fernandez 1999) purchased from Novagen, Inc (Madison, WI).



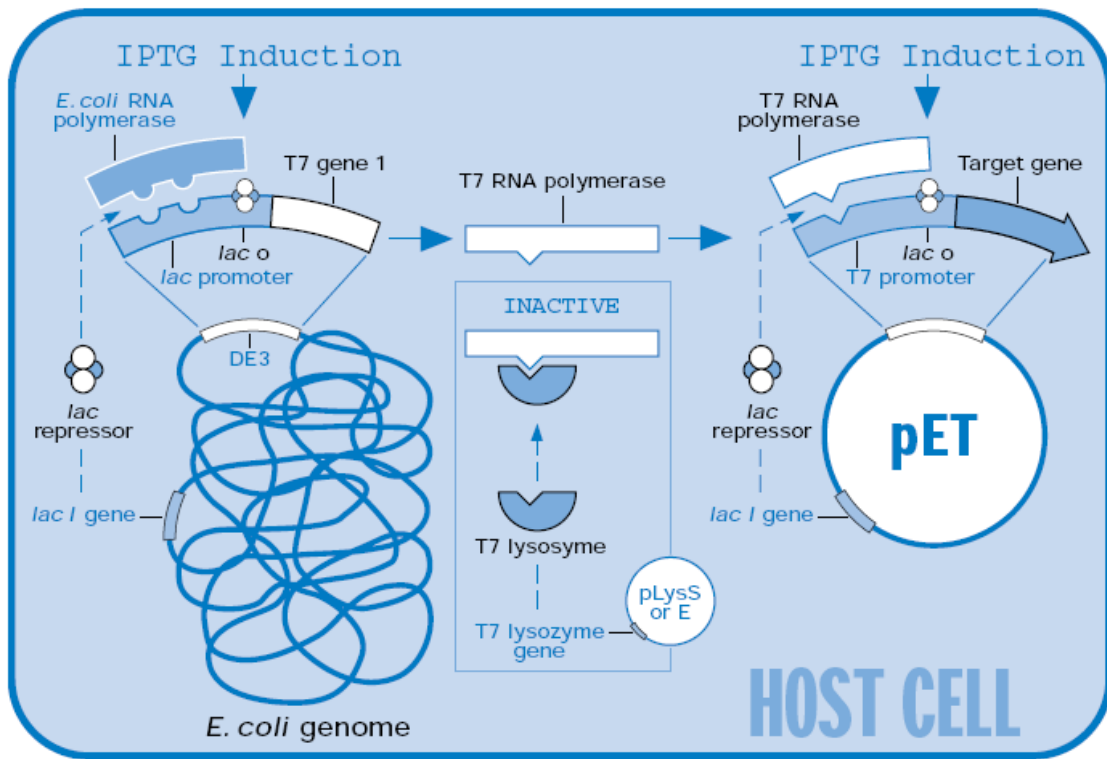
**Table 5:** Overview of the pET system process (Novagen 2006).

<p>Overview of the pET system process</p> <ol style="list-style-type: none"><li>1. Choose a pET vector<ul style="list-style-type: none"><li>- Application of the expressed protein</li><li>- Specific information known about target protein</li><li>- Cloning strategy</li><li>Solubility and cellular localizations</li><li>Fusion tags: need for tag removal</li><li>Regulation of protein expression (i.e., promoter and expression strain)</li></ul></li><li>2. Prepare pET vector<ul style="list-style-type: none"><li>- Digest with restriction enzyme(s) and dephosphorylate (or use LIC vector)</li><li>- Gel-purity (or use LIC vector)</li></ul></li><li>3. Prepare insert DNA<ul style="list-style-type: none"><li>- Plasmid and/or PCR DNA</li><li>- Restriction digest or generate LIC overhangs</li><li>- Gel-purity</li></ul></li><li>4. Clone insert into pET vector<ul style="list-style-type: none"><li>- Ligate or anneal insert with pET vector</li><li>- Transform into non-expression host (e.g., NovaBlue)</li><li>- Identity positive clones; colony PCR, prepare plasmid DNA, verify reading frame by sequencing</li></ul></li><li>5. Transform into expression host<ul style="list-style-type: none"><li>- Transform host carrying T7 RNA polymerase gene (<math>\lambda</math>DE3, lysogen) or non-DE3 host compatible with <math>\lambda</math>CE6 infection</li></ul></li><li>6. Induce and optimize expression of target protein<ul style="list-style-type: none"><li>- Determine time course and temperature for expression and temperature for expression in total cell and subcellular fractions; analyze solubility and activity;</li><li>- Detection target protein by SDS-PAGE, Western Blot, quantitative assay</li></ul></li><li>7. Scale-up culture size</li><li>8. Extract target protein<ul style="list-style-type: none"><li>- Detergent methods</li><li>- Mechanical methods</li></ul></li><li>9. Purify target protein<ul style="list-style-type: none"><li>- Affinity purification</li><li>- Cleave tags and remove protease (if desired)</li></ul></li></ol>
---

The desired protein (annexin V) can easily be expressed from pET recombinants by induction of the T7 or T7*lac* promoter (Figure 24) (Fernandez 1999). According to the fact that the expression using this system occurs in high levels, the protein purification is a relatively easy and straightforward process, with few chromatographic steps (2 or 3).

The use of fusion tags can facilitate detection and purification of the desired protein. The His-Tag<sup>®</sup> sequence reveals a useful character for protein purification. Proteins attached to this tag can be purified under a denaturing state that is extremely appropriate for proteins expressed as inclusion bodies (Fernandez 1999).

In the protein expression step, a recombinant plasmid is transferred to an *E. coli* strain containing chromosomal copy of the gene for T7 RNA polymerase (Fernandez 1999).



**Figure 24:** Control elements of the pET system. Induction of protein expression in the pET system (Novagen 2006).

The induction step should be performed at 30 °C instead of 37 °C, since proteins accumulate as inclusion bodies at 37 °C. It is often possible to obtain soluble and active proteins using a constant temperature of 30 °C.

The pET-30 Ek/LIC vector contains a strong T7 *lac* promoter (Fernandez 1999), an optimized ribosome binding site (RBS) (Glick 1998), the coding sequence for the Ek protease cleavage site (AspAspAspAspLys↓) and a multiple cloning site that contains restriction enzyme sites to facilitate insert transfer (Figures 25, 26, 27, 28 and 29) (<http://www.dinalfa.com>). The coding sequence for the HRV 3C protease cleavage site (Leu Glu Val Leu Phe Glu / Gly Pro) was inserted by graduate student Naveen Palwai in Dr. Harrison's laboratory.

Plasmids are genetic elements derived from bacteria that can carry some genes of interest, and useful since they can be present in thousands of copies. The multiple cloning sites are used to genetically introduce the genes that code for a specific protein.



**Figure 25:** 6x-His-Protein (N-terminus) schematic diagram.

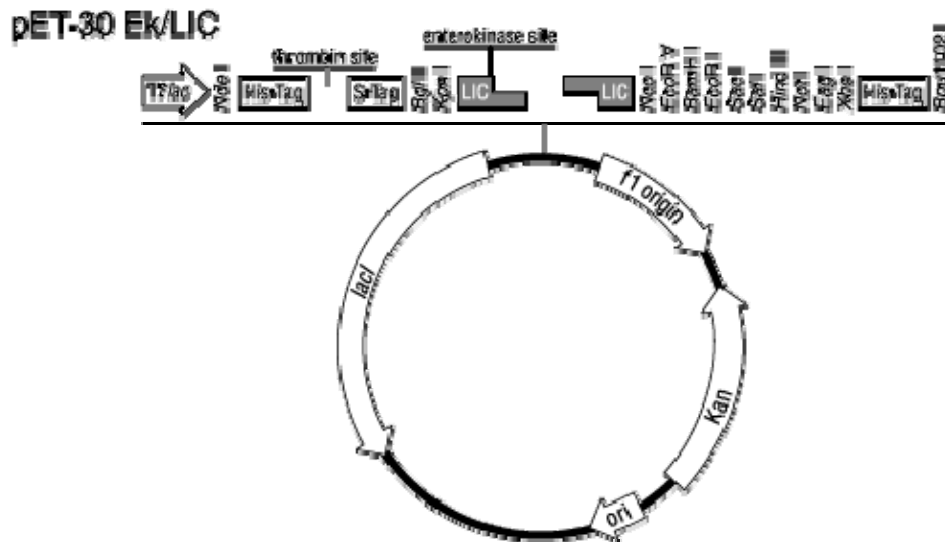


Figure 26: pET-30 Ek/LIC vector map (Novagen Catalog, 2006).

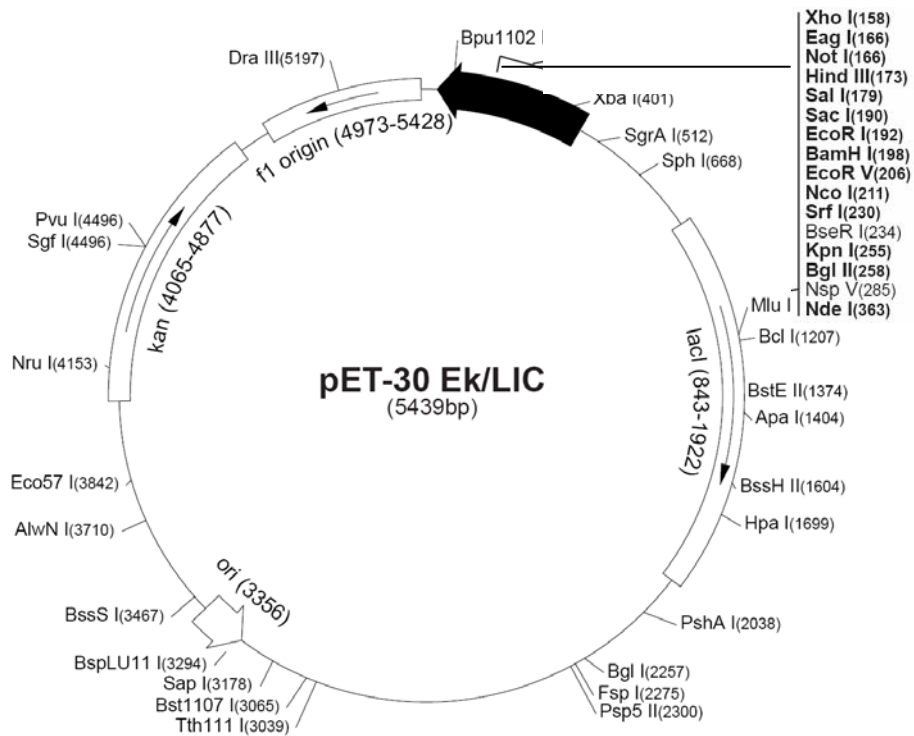


Figure 27: The pET-30 Ek/LIC vector map. General features of the pET-30 Ek/LIC vector (Novagen Catalog, 2006).

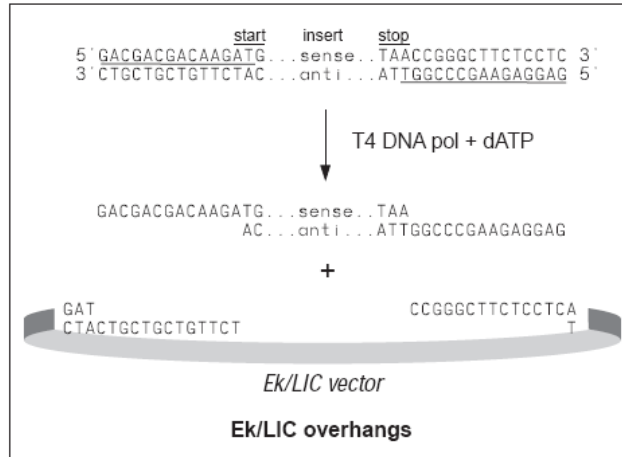


Figure 28: Ek/LIC overhangs (Novagen 2006).

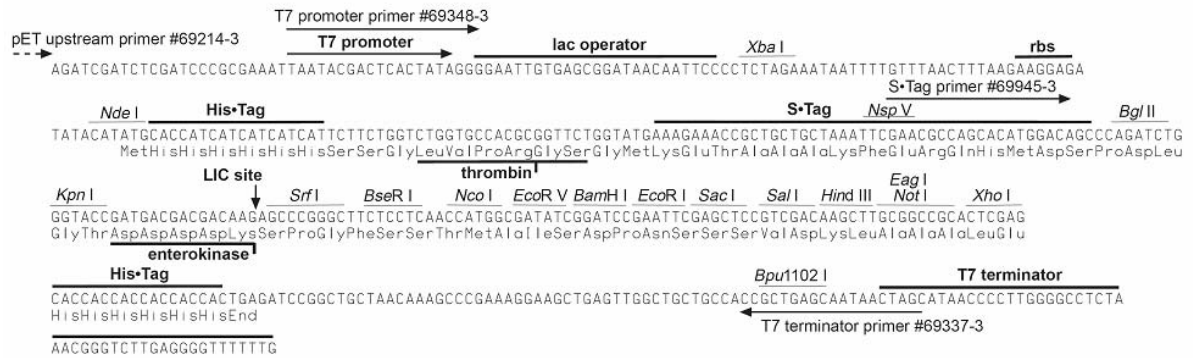
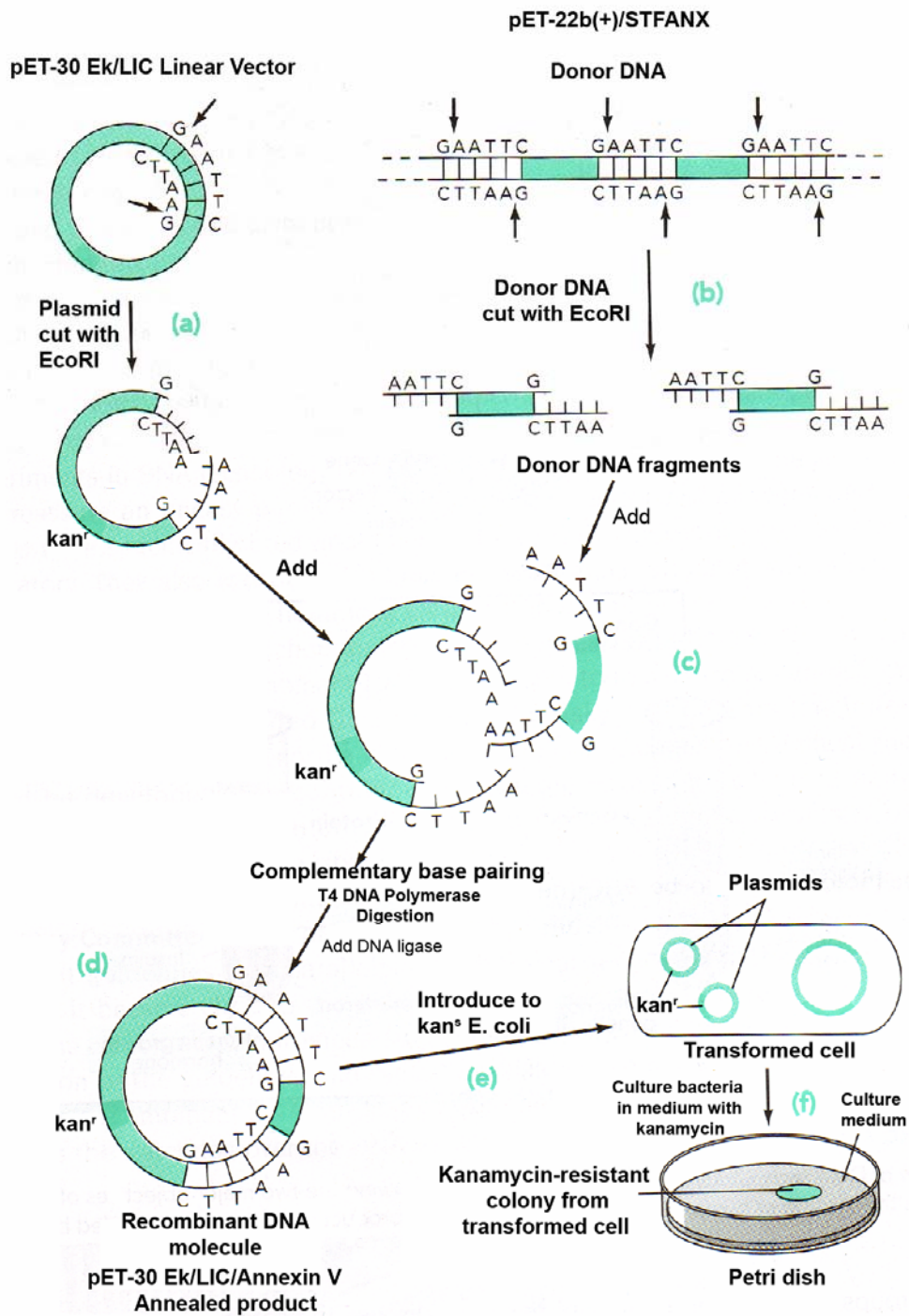


Figure 29: pET-30 Ek/LIC cloning/expression region (Novagen 2006).

The plasmid encoding for annexin V was previously prepared in the laboratory, and it was used on this specific study. The plasmid construction is described in the Ph. D. thesis of Naveen Palwai. Basically, the gene for annexin V was amplified by PCR (Dieffenbach 2003) from pET-22b(+)*STFANX* (parental plasmid – template), followed by digestion by T4 DNA polymerase (generation of sticky ends) and then annealing to linear pET-30 Ek/LIC vector (Figure 30). The parental plasmid (pET-22b(+)*STFANX*) contains annexin V-soluble tissue factor fusion gene.



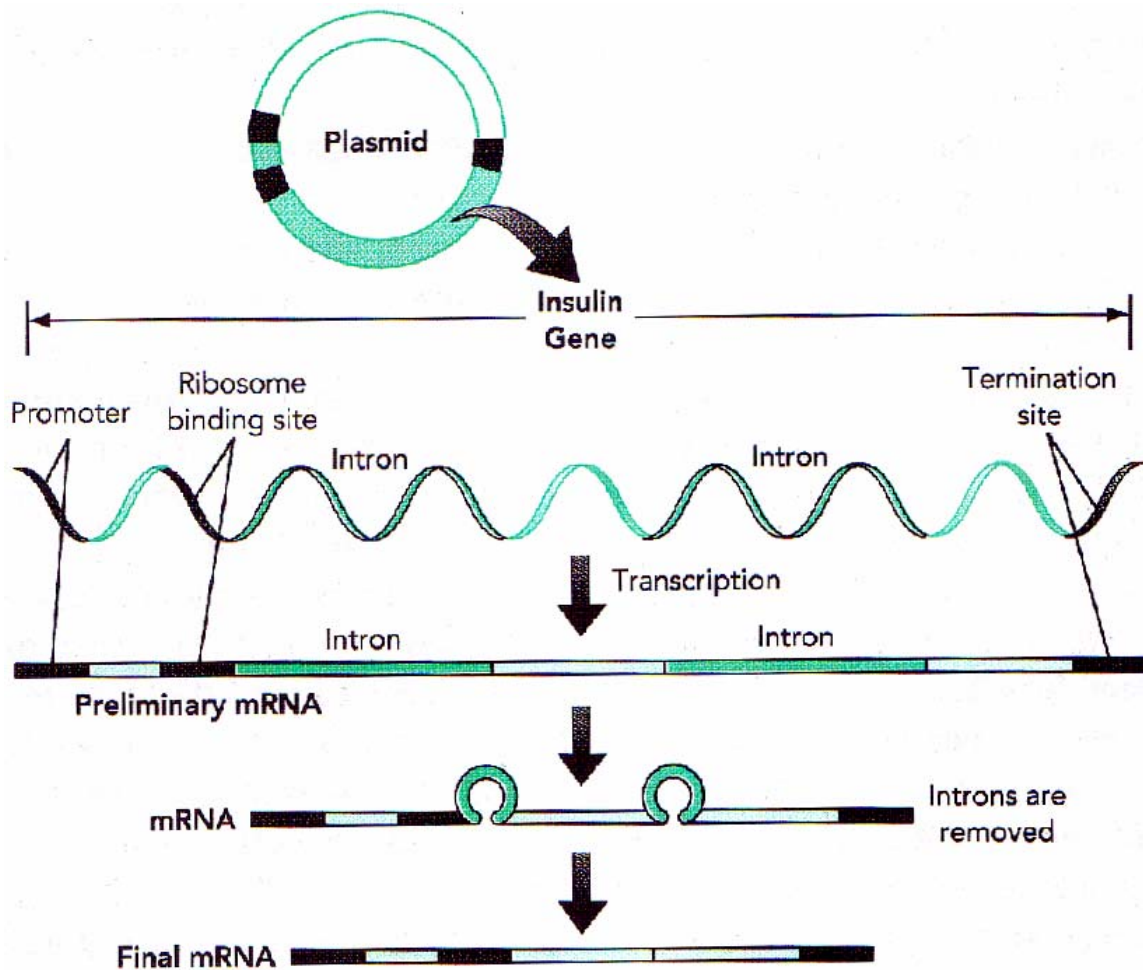


**Figure 30:** Overview of the cell transformation. **(a)** Plasmid pET-30 Ek/LIC is opened with the restriction enzyme *Eco*RI at the points shown by the arrows. The plasmid contains the gene for kanamycin resistance designated *kan*. **(b)** Donor DNA from the

pET-22b(+)/STFANX is also treated with *EcoRI*, and fragments of foreign DNA are obtained. Note that *EcoRI* acts at the same recognition sites in the plasmid vector and the donor DNA. **(c)** Fragments of donor DNA are combined with the opened plasmids, and complementary base pairing takes place. **(d)** When DNA ligase is added, the plasmid closes and a recombinant DNA molecule is formed. **(e)** The plasmids are introduced to kanamycin-sensitive ( $\text{kan}^S$ ) *E. coli* cells by treating the cells with calcium compounds. The *E. coli* cells are transformed. The plasmids multiply within the cells and encode the proteins specified by the annexin V DNA. **(f)** When bacteria are cultivated in medium containing kanamycin, the cells containing recombined plasmid grow and form colonies. This is because they possess the gene for resisting kanamycin. Bacteria containing the normal plasmids do not possess the kanamycin-resistance gene and fail to form colonies in the medium. Adapted from (Alcamo 2001).

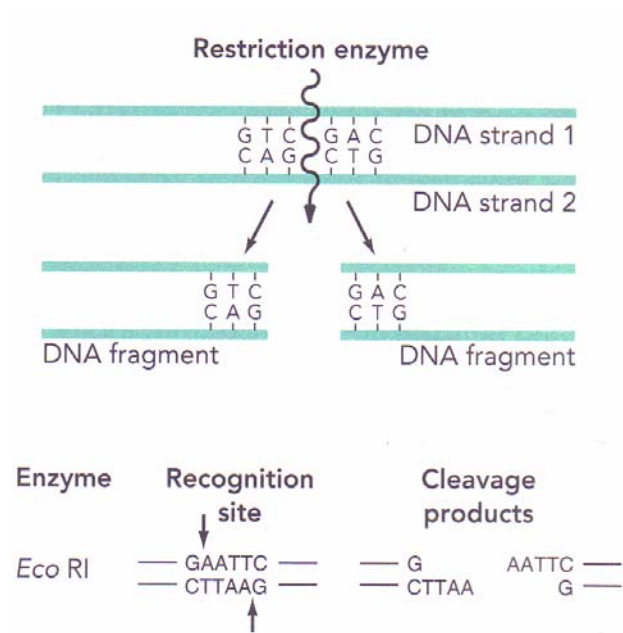
The plasmid carries some genes that can be expressed in the bacteria; however, it usually remains separate from the bacterial chromosome. Plasmids have several important characteristics that are essential to a successful expression protocol. Generally, a simple exposure to the bacteria is enough, since plasmids are highly infective. Plasmids generally replicate and are passed on to daughter cells along with the chromosome. Plasmids are modified natural vectors used for cloning and expressing proteins. However, they are modified since the unnecessary elements are removed, and they are more compact and efficient (Rodriguez 1987).

A polylinker called a multiple cloning site (MCS) can be defined as a region with restriction sites for inserting the DNA (Figure 27). The replication origin (*ori*) is the site that permits the initiation of the replication (Figure 31).

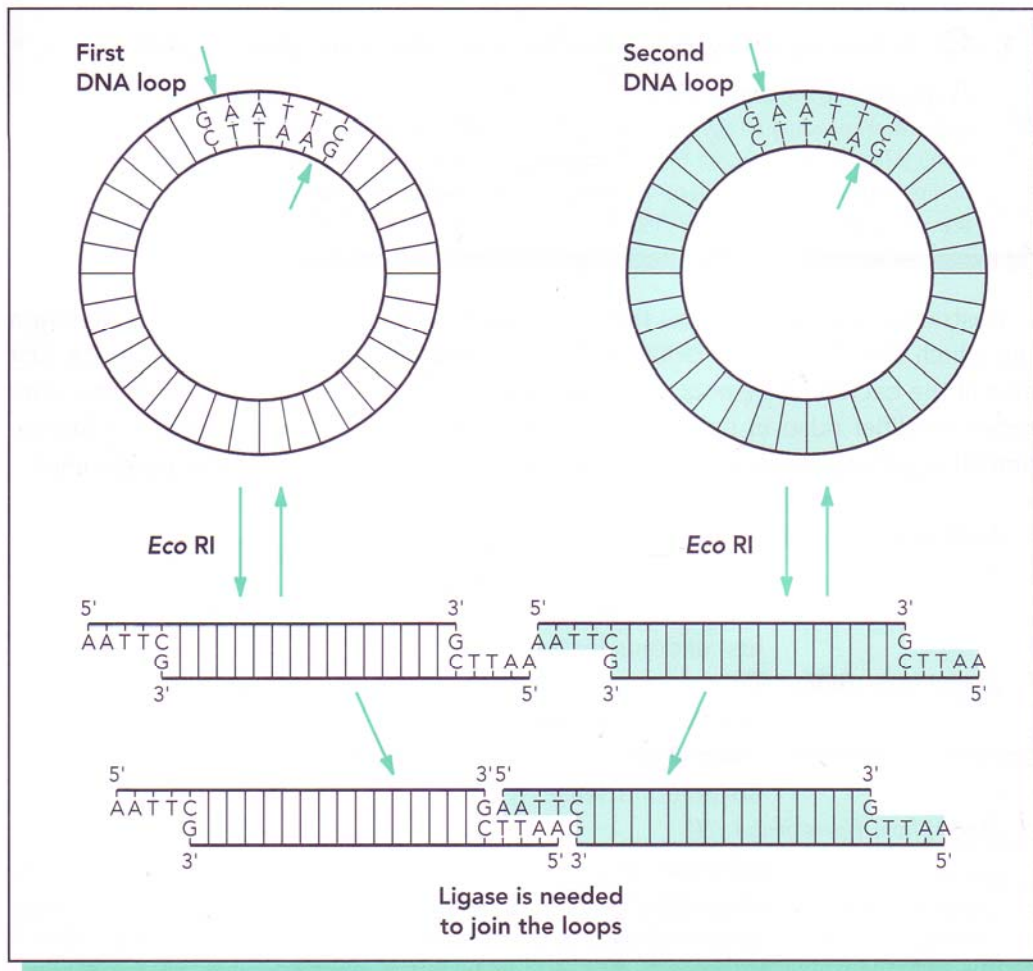


**Figure 31:** Essential features of a useful vector such as the plasmid that encodes insulin. The plasmid contains sites to initiate mRNA formation (the promoter); it also has sites to encourage binding to the ribosome and to terminate mRNA production (the terminator). In eukaryotic cells, the preliminary mRNA molecule is modified by removing the introns to form the final mRNA molecule. Adapted from (Alcamo 2001).

A restriction enzyme (or restriction endonuclease) is an enzyme that recognizes and cuts DNA only at a particular sequence of nucleotides (Glick 1998; Russell 1998; Lodish 1999; Kreuzer 2001). According to the incision type, the enzymes can produce blunt ends (cleaves both ends) or sticky ends (cleaves only one strand) (Figure 32). The complementary strand from the DNA insert will “match” the sticky ends, and they will insert in the plasmid followed by the ligation of the strands (T4 DNA ligase action) (Glick 1998; Russell 1998; Lodish 1999) (Figure 33).



**Figure 32:** Restriction enzyme. Activity of a restriction enzyme (also called endonuclease). A restriction enzyme cuts through the two strands of a DNA molecule to produce two fragments. The restriction enzyme shown at the top leaves blunt ends in the DNA fragments. The recognition site of the *Eco*RI restriction enzyme is illustrated on the figure at the bottom. Arrows show where the restriction enzyme function. Note that at a particular recognition site the sequences of nucleotides of the two DNA strains run in opposite directions. This symmetry is called twofold rotational symmetry. Also, note that the enzyme produce fragments that dangle. These dangling ends are desirable and essential to gene-combining techniques (Alcamo 2001).



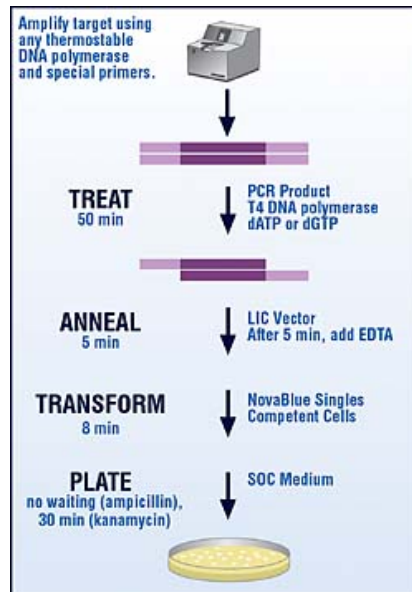
**Figure 33:** Construction of a recombinant DNA molecule from two unrelated plasmids. The restriction enzyme *EcoRI* cleaves the DNA of both plasmids because; even though they are unrelated, the plasmids have the identical recognition sites in their DNA. The circular plasmids now become linear. At this point the exposed ends join with each other, and the two linear molecules can rejoin with one another to form a single, long DNA molecule. Also, the ends can join to one another to form a large loop. The union is catalyzed by DNA ligase. Unions such as these are the basis for synthetic genetic recombinations (Alcama 2001).

The restriction maps of plasmid are very useful because they show where all restriction enzymes will do the cleavage process (Appendix C and Figure 27) (Russell 1998).

### 6.2.2. Cell transformation

The process characterized by the introduction of plasmids into *E. coli* is called transformation (Figure 34). Transformation is the genetic recombination process that is characterized by the acquisition of DNA fragments from the local environment by the bacteria, expressing the proteins coded by the genes present in those fragments (Bloom 1995; Glick 1998; Russell 1998; Lodish 1999; Griffiths 2000; Kreuzer 2001; Cooper 2004).



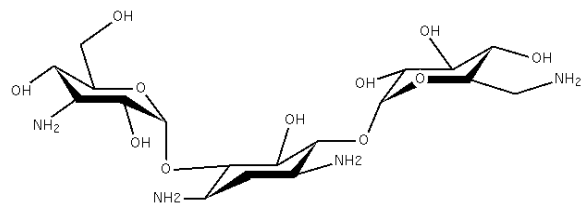


**Figure 34:** Overview of cell transformation (Novagen Catalog, 2006).

### 6.2.3. Cell expression

Because in this work a plasmid previously prepared was used, the first stage in the cell expression was the culture growth. The main goal of protein expression is the maximization of pure protein produced. In order to increase the yield of the production system, adequate conditions should be assured. Aeration is an extremely important variable in order to provide an effective oxygen supply to the cells (Glick 1998). Another important and necessary chemical compound is the antibiotic.

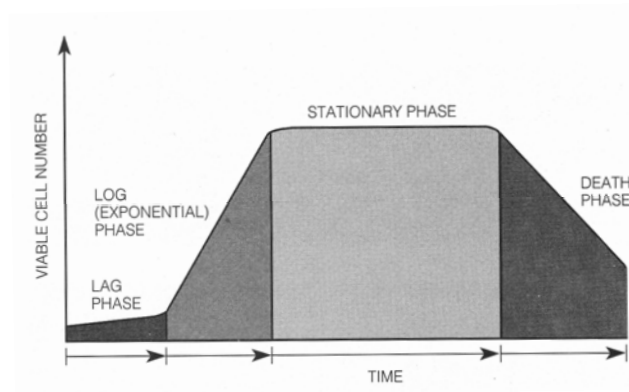
Antibiotics are chemicals that kill microorganisms. However they do not present toxicity to eukaryotic organisms. An example of an antibiotic, kanamycin, is shown in Figure 35. There are some genes encoding resistance to the antibiotics. The plasmid, phage vectors and cells that carry the vector are capable of growing and consequently form colonies in the presence of the specific antibiotic, as long as the correct antibiotic resistance gene is present (Rodriguez 1987).



**Figure 35:** Kanamycin (<http://www.wikipedia.org/>).

The use of antibiotics during the cell culture is extremely important in order to avoid culture contamination. Another aspect regarding the use of specific antibiotics is that they promote the selectivity in the cell culture, allowing only the growing of the bacteria that carries the eligible vector. Contaminant bacteria or non-transformed cells will not be able to survive in the medium.

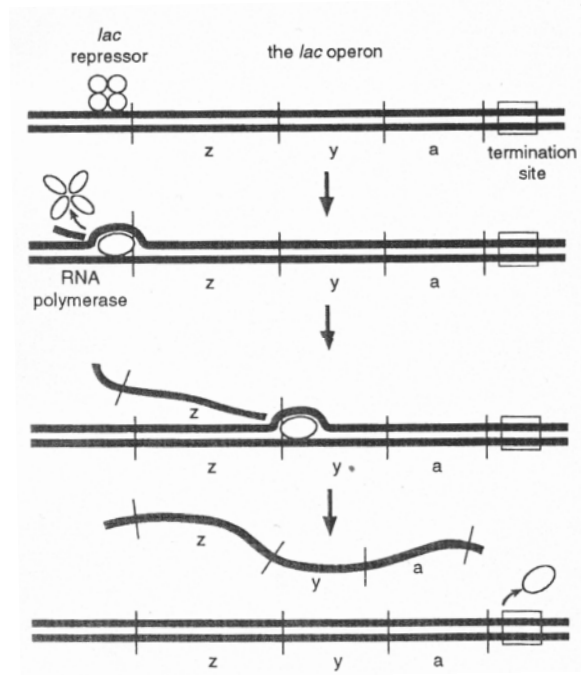
Regarding the importance of a high yield, the host cells should be in the exponential growth phase (Figure 36), in the cell induction stage. Generally, it is possible to scale up the process by successively inoculating a small culture – cultivated overnight using a fresh and rich medium (e.g., Luria Bertani (LB) (Bloom 1995)) – into a large culture (10-fold dilution).



**Figure 36:** *E. coli* growth curve (Bloom 1995).

In our study, we used the pET vector system commercialized by Novagen, Inc.. Due to, to its important characteristics, this system is considered an excellent system for use in bacteria transformation. The kanamycin resistant marker, *lacI* gene (*lac* repressor protein), T7 transcription promoter (specific for phage T7 RNA polymerase), *lac* operator region 3' to the T7 promoter, multiple cloning site (polylinker region) downstream of the promoter are important elements present in this system (Glick 1998).

*E. coli* contains a *lac* operon (that carries three genes: *lac Z*, *lac Y* and *lac A*) (Figure 37). The *lac* repressor inhibits the transcription during the cell growth in a rich medium. It binds to an operator (single site) upstream of *lac Z* and does not allow RNA polymerase to bind to the promoter (Figure 37) (Glick 1998; Fernandez 1999; Griffiths 2000).

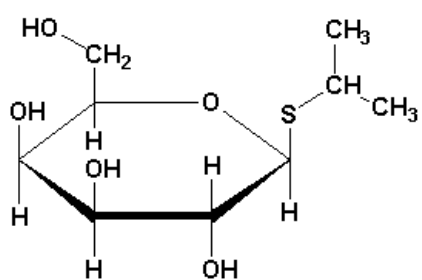


**Figure 37:** The first line shows the genes of the *lac* operon, *lacZ*, *lacY*, and *lacA*. Transcription is repressed by the *lac* repressor. In the next line, inactive repressor is no longer bound to the operator, and the genes are being transcribed by RNA polymerase. In the third line, RNA polymerase transcribes the operon. Finally, RNA polymerase is detached from the DNA and the nascent transcript (Ausubel 1987).

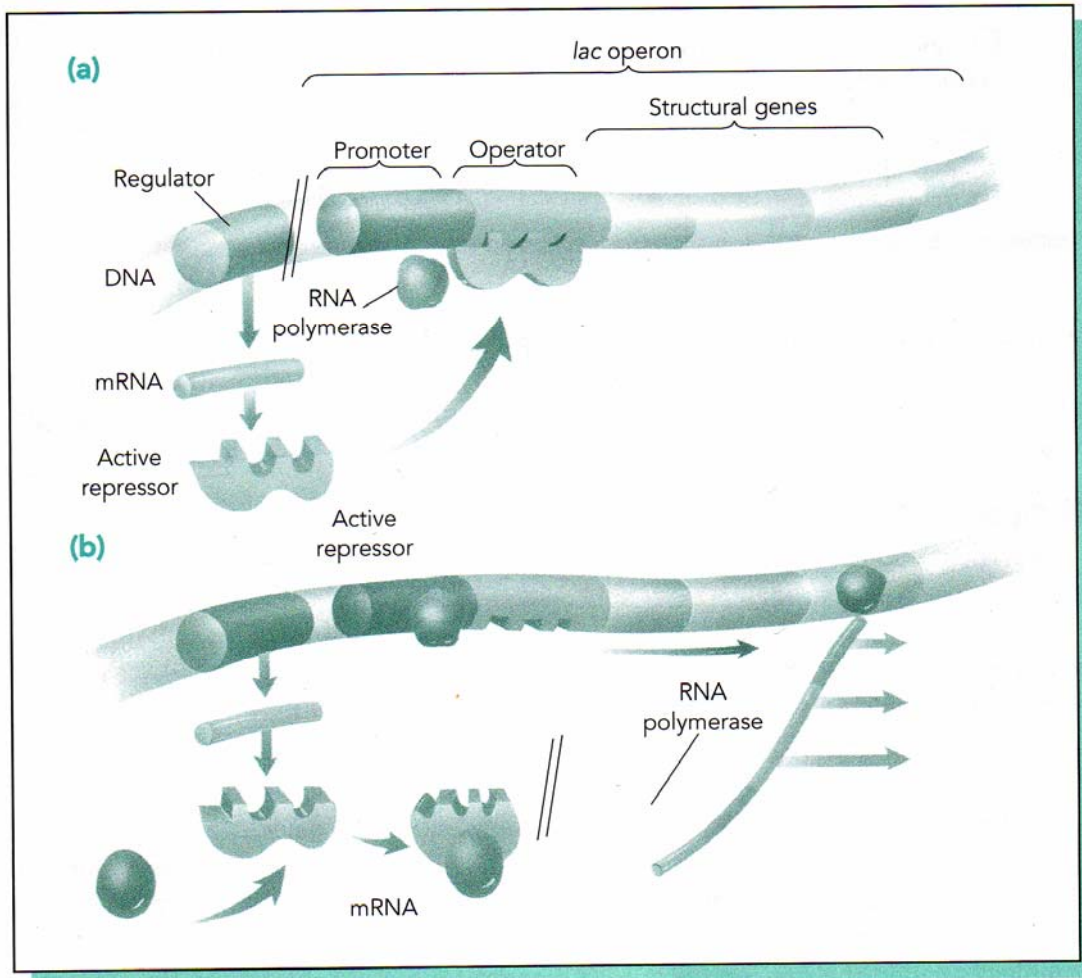
IPTG (isopropyl-beta-D-thiogalactopyranoside) (Formula  $C_9H_{18}O_5S$ , molecular weight: 238.3) (Figure 38) is a reagent that is responsible for the induction of beta-galactosidase enzyme (Cooper 2004). This enzyme is useful because when it binds to the *lac* repressor, it inhibits it, thus promoting the lactose utilization (Fernandez 1999).

In cloning experiments, IPTG is used to induce *lacZ* gene expression. In *E. coli*, the IPTG-inducible protein expression is regulated primarily by the T7 *lac* promoter. When the IPTG is added to the solution, *lac* repressor does not bind the operator. This is important since the RNA polymerase synthesizes a single mRNA that encodes *lacZ*, *lacY* and *lacA* (Figure 39).





**Figure 38:** Isopropyl-beta-D-thiogalactopyranoside (IPTG) structure  
(<http://www.molecula.com/IPTG.gif>).



**Figure 39:** An example of control of gene expression as exemplified by the *lac* operon. **(a)** A regulator gene encodes an mRNA molecule, whose code is translated to a repressor protein. The repressor protein binds to the operator gene in the regulatory region of a gene. The enzyme RNA polymerase normally passes through the regulatory region, but with the repressor protein in place, the enzyme cannot move through the regulatory region, and gene transcription is halted. **(b)** Certain molecules called inducers can remove the repression by uniting with the repressor protein. In the *lac* operon, a lactose molecule unites with the repressor protein. This union inactivates the repressor and removes it from the regulatory site. Now the RNA polymerase is free to begin transcription of the gene, and enzyme molecules result (Alcamo 2001).

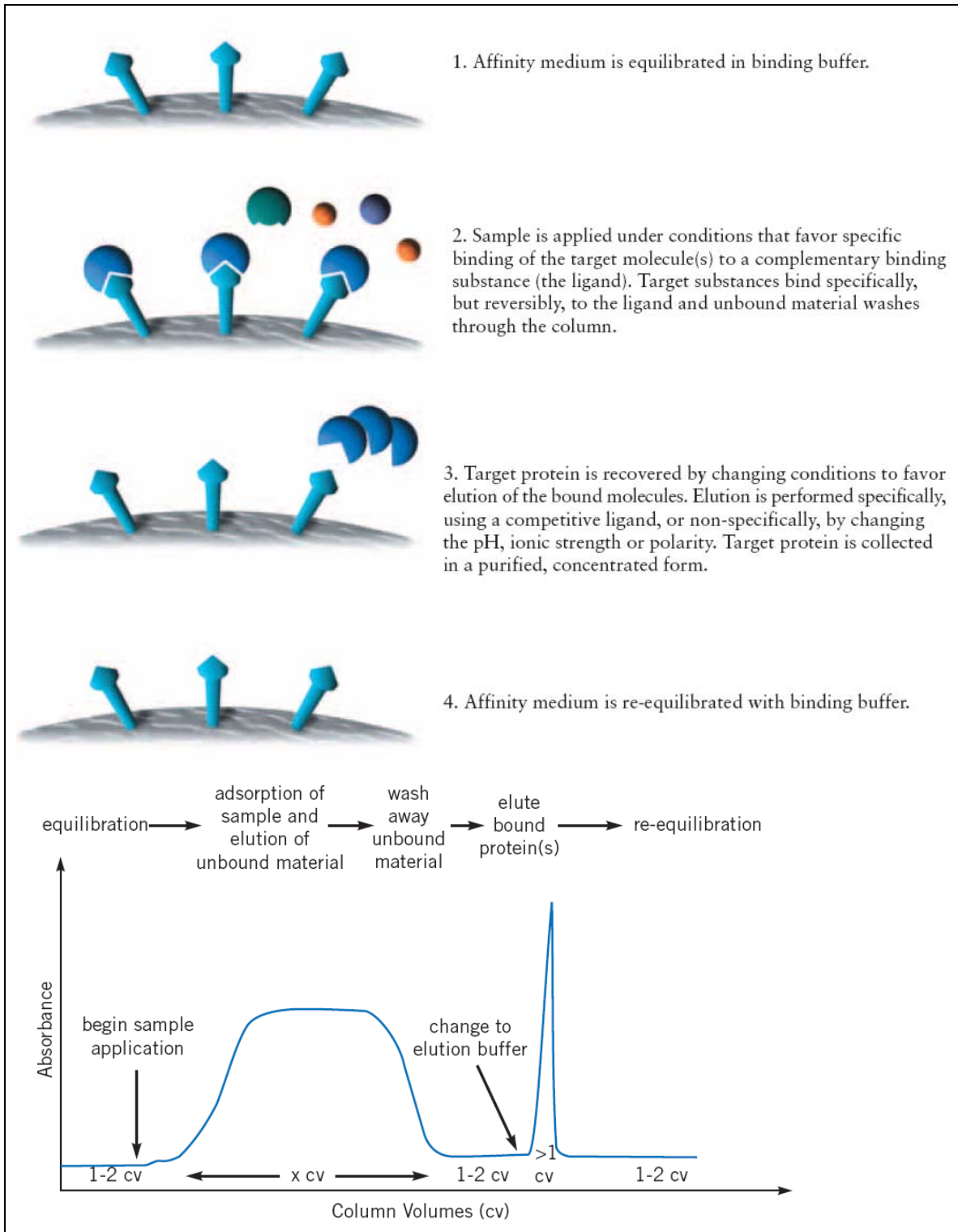
After the adding of the inducer agent, the cell incubation is performed at 30 °C for 5 hours, while providing an excellent O<sub>2</sub> supply.

#### 6.2.4. Protein purification

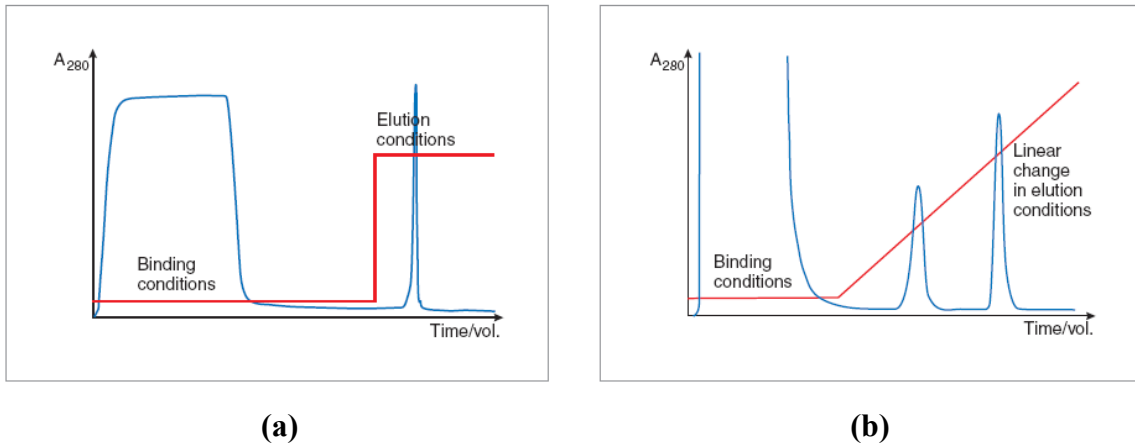
The protein purification requires several steps, beginning with the cell culture, followed by induction and expression of the heterologous protein, host cell lysis (e.g., sonication), centrifugation (preclearing) and finally purification and separation of the recombinant protein obtained from the cell proteins of the host organism.

When the expression system does not have the capacity to secrete protein into the culture medium, it is necessary to perform a cell lysis in order for the purification of the protein to be possible. To promote the cell lysis, several chemical and physical methods are available. The utilization of ultrasounds is one of the techniques preferentially selected. The energized tip of the sonicator is immersed in the solution, causing the transfer of energy to the cellular suspension, leading to the destruction of cell walls and cell membranes. Other methods can also be used such as thermic shock, high pressure lysis and use of detergents (e.g., SDS) (Glick 1998).

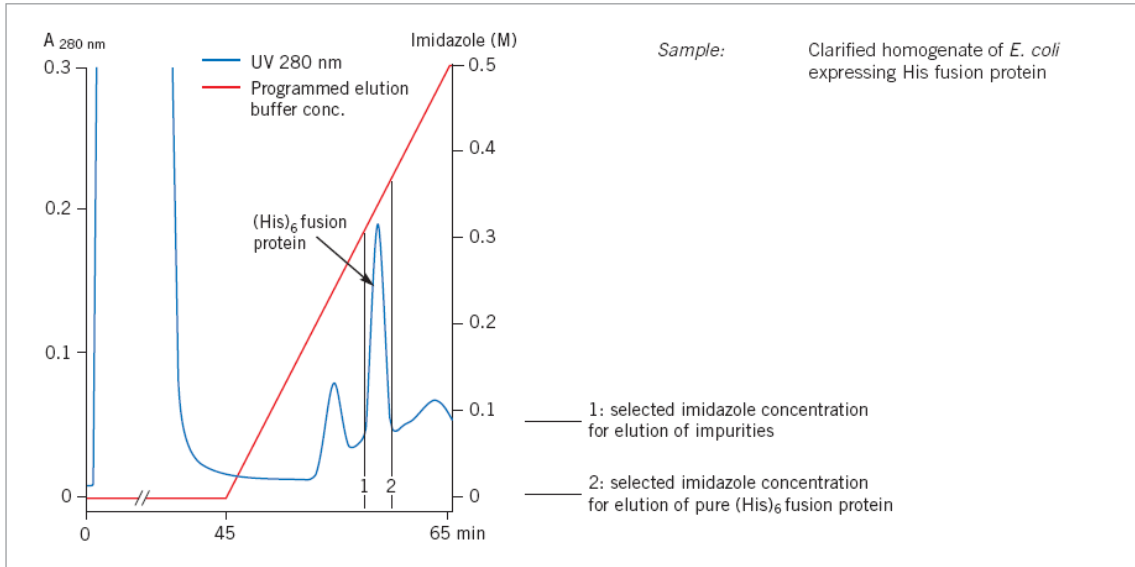
The use of a chromatography system is required in the protein purification stage. This method is a simple procedure based on molecular recognition of an inherent recognition site (that can be a natural or engineered molecule). This system permits the protein elution in the purified form (Scopes 1982). Chromatography is an experimental technique that is used to separate mixtures involving the passing of a mixture through a stationary phase, that separates the desired molecule from contaminant molecules allowing the desired molecule to be isolated (Figures 40, 41, 42 and 43) (Scopes 1982; Harrison, Todd et al. 2003).



**Figure 40:** Typical affinity purification (Amersham Biosciences 2002).



**Figure 41:** (a) Step elution. (b) Gradient elution (Amersham Biosciences 2002).



**Figure 42:** Gradient elution of a  $(\text{His})_6$  fusion protein. Adapted from (Amersham Biosciences 2002).

In order to carry out metal-chelate affinity chromatography (Table 6), some affinity tags or tails that bind to the column are often utilized, making possible the protein purification. The His-tag is an example of an affinity tag (Figure 40).

The chromatography method requires five different steps:

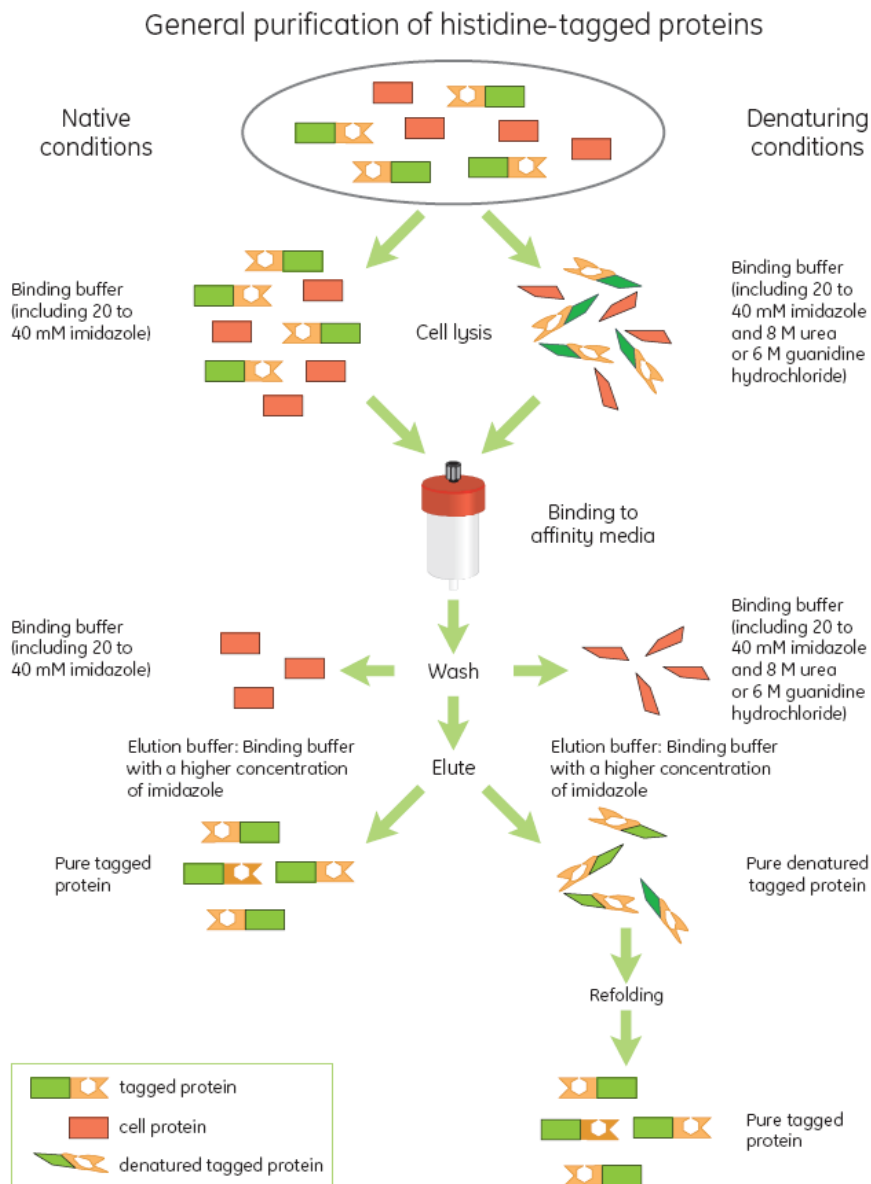
- a. activation of the matrix;
- b. coupling of ligands;
- c. adsorption of the protein;
- d. elution of the protein;
- e. regeneration of the affinity matrix.

**Table 6:** Biomolecules purified by affinity chromatography (Bailon 2000).

---

1. Antibodies and antigens	9. Lectins
2. Enzymes and inhibitors	10. RNA and DNA (genes)
3. Regulatory enzymes	11. Bacteria
4. Dehydrogenases	12. Viruses and phages
5. Transaminases	13. Cells
6. Hormone-binding proteins	14. Genetically engineered proteins
7. Vitamin-binding proteins	15. Others
8. Receptors	

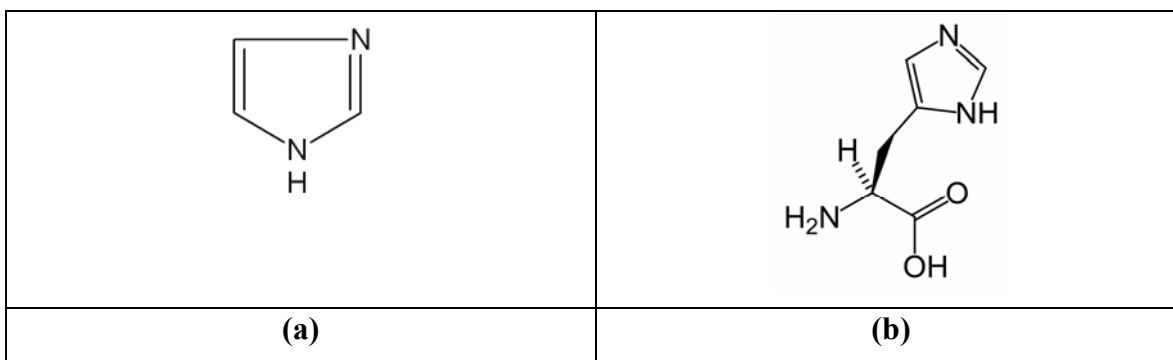
---



**Figure 43:** Overview of a typical purification flow scheme for (histidine)<sub>6</sub>-tagged proteins using HisTrap HP including purification under denaturing conditions. To obtain the highest possible purity, imidazole is included both in the sample before loading to the column and during the wash with binding buffer (GE-Healthcare 2006).



According to the basic principle that all biological molecules have affinity for a particular particle, in 1987 a new chromatography method was developed known as His-tag chromatography. Inside of the chromatography column, there is a resin with affinity groups (usually NTA and carboxylic groups) that permit the specific binding of certain molecules. Imidazole is a component of the amino acid histidine that presents high affinity to  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  (Figure 44). The hexahistidine sequence present in the genetically engineered protein will bind to the metal groups present in the resin. This affinity will provide an efficient method that eliminates all unwanted molecules, i.e., molecules that do not present the His-tag sequence (using the appropriate wash buffer).



**Figure 44:** (a) Imidazole chemical structure (b) Histidine chemical structure  
(<http://www.wikipedia.org/>)

This type of chromatography (IMAC – immobilized metal ion affinity chromatography) using specific tags like the histidine tag, is considered the most efficient method. The His-tag can be attached to the N- or C-terminal of the protein.

### 6.2.5. Protein determination

The protein concentration was determined using the Bradford protein assay, which is a chromogenic (color-forming) assay used to determine the protein concentration using a dye (Coomassie Brilliant Blue R250 ( $C_{43}H_{44}N_3O_7S_2Na$ )), that when bound to the protein turns blue.

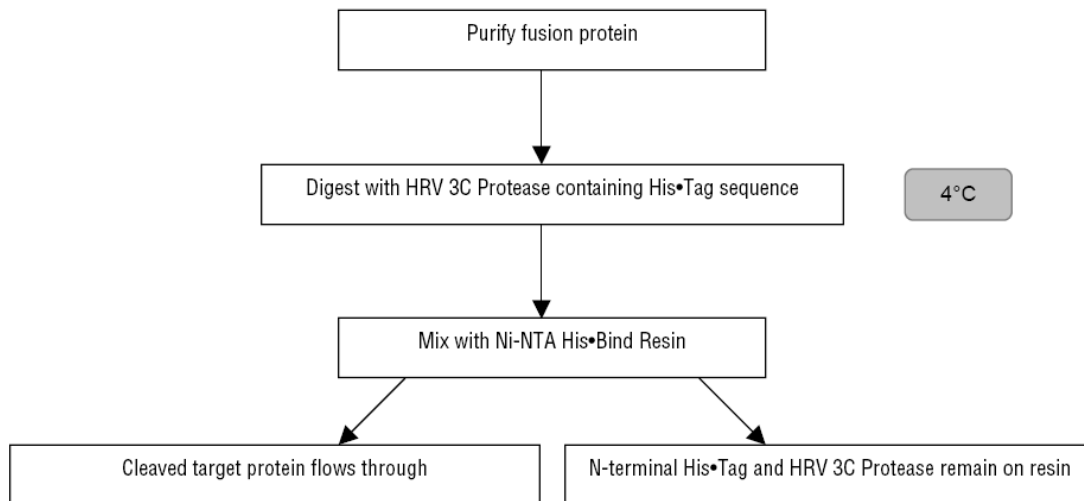
This specific dye binds to the hydrophobic regions of the protein structure, causing a shift in the absorption maximum of the dye. This shift is from 465 nm (red) to 595 nm (blue) in acidic solutions. The standard curve is used to estimate the concentration in each of the unknown samples.

A standard curve (absorbance vs. protein concentration) can be obtained using known protein concentrations (Appendix A.2.2). An equation can be obtained from the obtained data, allowing the determination of the protein concentration of other proteins.

There are several methods for determining the protein concentration of a specific sample. Biuret, Lowry, BCA and Bradford assays are examples of those methods. However, the most used method is the Bradford protein assay.

### 6.2.6. HRV 3C protease

Since the annexin V recombinant protein has a His-tag sequence attached, an enzyme (HRV 3C protease) is used in order to make it possible to elute the pure protein. HRV 3C protease is a recombinant form of the 3C protease from human rhinovirus type 14, and it is a highly purified His-tag fusion protein. It is possible to obtain a pure protein without any vector-encoded sequences due to the site-specific cleavage promoted for that enzyme (Figure 45). Robust activity at 4 °C, small size (22 kDa), high specific activity, and His-Tag fusion are some important characteristics that contribute to the rapid removal of His-Tag fusions as well as the protease by digestion followed by immobilized metal affinity chromatography (IMAC). The cleavage site, LeuGluValLeuPheGln/GlyPro, is recognized by the HRV 3C Protease.



**Figure 45:** HRV 3C protease experimental outline (Novagen 2004).

## 7. Protein purity analysis – gel electrophoresis

Molecular analytical separation is performed using a technique called as gel electrophoresis based on physical properties such as size, shape or isoelectric point. Electrophoretic separation is the method most used to estimate the size of proteins, nucleic acid fragments, DNA or RNA. This technique requires a gel, which basically is a matrix formed by a crosslinked polymer with a composition and porosity that is adjusted according the weight and composition of the target of the analysis. At neutral pH, nucleic acids present a negative charge migrating towards the positive electrode.

Generally a solution with molecules of various known sizes (marker or *ladder*) is injected on the first lane that is used in order to compare with the other lanes. This allows for the being determination of the molecular weight analysis of a particular sample.

The gel is cast between two sealed glass plates and collocated in an electrophoresis tank immersed in a buffer solution. The samples are injected in the gel lanes. DNA samples migrate from the negative pole (cathode) to the positive pole (anode), since the nucleic acids have a negative charge at neutral pH. This gradient is provided by an electric field. The gel has a separating property, i.e. the larger molecules have a low migration rate when compared to the smaller molecules. The molecules with identical sizes will move together creating bands that can be analyzed using several methods, such as Coomassie Blue staining, fluorescence, autoradiography, silver staining and imunoblotting. The relation between the migration rate and the migrated length is inversely proportional. DNA electrophoresis can be prepared using an agarose or acrylamide gel; however, the acrylamide gel allows for a better fragment resolution that can be used for DNA sequencing (Rothe 1994).

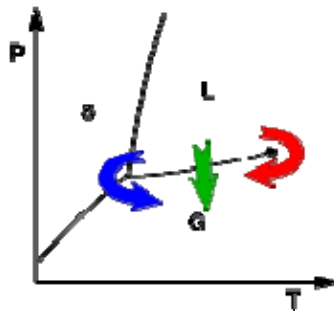
### 7.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a useful analysis technique that permits the estimation of molecular masses and relative abundance of unknown polypeptides in a complex mixture (Rothe 1994; Sanchez 2004). It allows the detection of isoenzymes or complex proteins. It is an

effective method used to evaluate the protein purity after the protein purification method. The protein purity can easily be analyzed by the gel electrophoresis technique (Rothe 1994; Sanchez 2004). SDS binds to the protein or peptide, causing it to denature and to be uniformly negatively charged. The acrylamide acts as a molecular sieve separating molecules of different lengths.

## 8. Freeze drying

Freeze drying or lyophilization, is a dehydration process characterized by three steps: freezing, primary drying and secondary drying. It is a method used to preserve a specific material; in our study it was used to preserve the annexin V protein. The process is initiated with the sample freezing followed by decreasing the surrounding pressure combined with adding enough heat to allow for the water sublimation in the sample (Figure 46). Freeze drying is contact drying that takes place at such a low vapor pressure in which the water temperature drops below the freezing point.

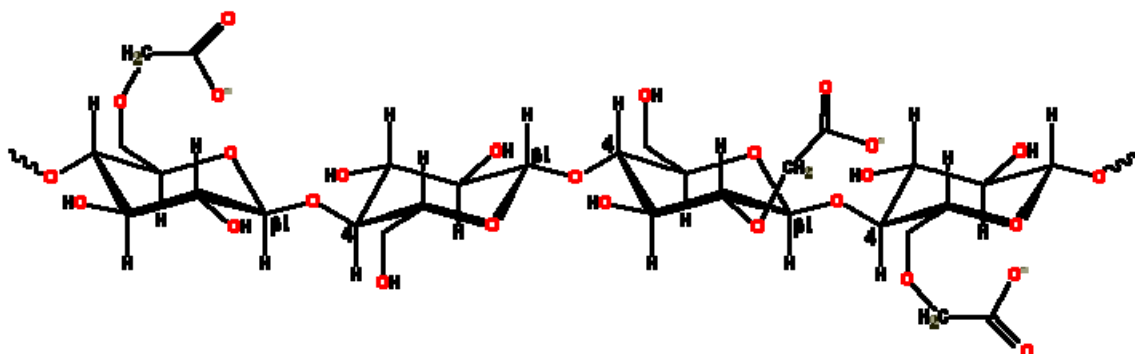


**Figure 46:** In a typical phase diagram, the boundary between gas and liquid runs from the triple point to the critical point. Freeze drying (blue arrow) brings the system around the triple point, avoiding the direct liquid-gas transition seen in ordinary drying (green arrow) (<http://www.wikipedia.org/>).



## 9. Attachment of annexin V to SWNTs using EDC and NHS

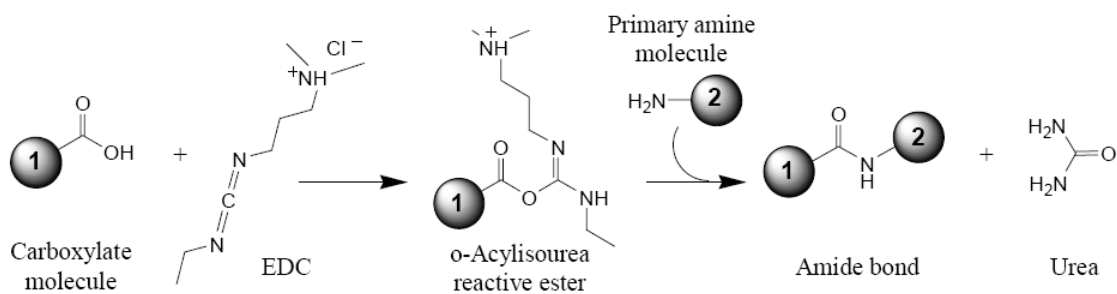
As an alternative to the adsorption of annexin V directly on SWNTs, a method involving carboxymethylcellulose (CMC) was evaluated. In this method, the SWNTs are first dispersed in the presence of the CMC (Figure 47) to give a SWNT-CMC complex. Excess CMC is removed by dialysis. The annexin V is covalently coupled to CMC adsorbed on the SWNTs using 1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Figure 48), which is a zero-length crosslinking agent used to couple carboxyl groups to primary amines.



**Figure 47:** Carboxymethylcellulose structure (<http://www.lsbu.ac.uk/water/hycmc.html>).

This crosslinker has been used in diverse applications such as forming amide bonds in peptide synthesis, attaching haptens to carrier proteins to form immunogens, labeling nucleic acids through 5' phosphate groups and creating amine-reactive NHS-esters of biomolecules. EDC reacts with a carboxyl to form an amine-reactive O-acylisourea intermediate. The intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. If this intermediate does not encounter an amine, it will hydrolyze and regenerate the carboxyl group.

The extent of this reaction can also be evaluated by direct measurements of the urea in solution.



**Figure 48:** One-step EDC reaction with carboxyl and amine-containing molecules. EDC reacts with a carboxyl group on molecule #1, forming an amine-reactive O-acylisourea intermediate. This intermediate may react with an amine on molecule #2, yielding a conjugate of the two molecules joined by a stable amine bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. (Pierce Catalog, 2006).

In the first stage, HRP was used instead of annexin V in order to test and optimize our protocol. The successful results are presented in the Results section of this dissertation.

# **CHAPTER II**

## **MATERIALS**

### Bacterial strains and plasmids

Plasmid pET-30 Ek/LIC, host strain *E. coli* BL21 (DE3) (protein expression host) and *E. coli* NovaBlue GigaSingles™ Competent Cells were purchased from Novagen (Madison, WI).

### Enzymes

HRV 3C Protease and T4 DNA Polymerase were acquired from Novagen (Madison, WI).

### DNA synthesis, purification and analysis

For the PCR amplification, primers were obtained from the Molecular Biology Resource Facility at Health Sciences Center – University of Oklahoma. The sequencing of all genes was performed at the Oklahoma Medical Research Foundation (OMRF) DNA sequencing facility using the primers previously mentioned.

### *E. coli* cell cultures media

NaOH, NaCl, tryptone, kanamycin and yeast extract were purchased from Sigma (St. Louis, MO).

### Cell transformation

A water bath (Water Bath 184, Precision® (Illinois, USA)), an incubator (Incubator VWR-1540 (VWR Scientific Inc., USA)), SOC medium and kanamycin (Sigma, St. Louis, MO) were used.

### Protein purification

The HisTrap chromatography column (5 ml) was purchased from GE Health Biosciences Corp. (Piscataway, NJ). Flow adapters were purchased from Bio-Rad (Hercules, CA). An ISCO UA-5 chart recorder and absorbance / fluorescence detector with a type-10 optical unit (Isco, Lincoln, NE) was used in the protein detection (280 nm) step. A peristaltic pump – Gilson MiniPlus 3 (Gilson Medical Electronics Inc., Middletown, WI) was used during the protein purification process. A Foxy Model 2200 Fraction collector was used to collect the samples. A Shimadzu UV-2100 PC UV-VIS Scanning spectrophotometer (Japan) was used to perform the absorbance measurements.

### Freeze drying / lyophilization

A Hetosicc Heto FD 2.5 (Heto Lab Equipment) freeze drying system was used connected to a Jouan Refrigerator cold trap (-105 °C) and TRIVAC (LH-Leybold, USA) vacuum pump.

### SDS-PAGE

A Bio-Rad mini-protean 3 cell gel electrophoresis unit (Bio-Rad, Hercules, CA) and an Integrated Separation Systems (ISS-empotech ISS 500) power supply were used.

### SWNT suspension preparation

Purified and freeze dried CoMoCAT SWNTs were provided by Southwest Nanotechnologies, Inc. Sodium cholate (NaC, from Sigma-Aldrich) and low-viscosity sodium carboxymethylcellulose (CMC, from Sigma-Aldrich) were used as dispersants. A CPX 750, Cole Parmer horn sonic dismembrator equipped with a microtip of 1/8" in diameter was used. The resulting suspension was centrifuged in an automatic centrifuge

(IEC Multi, Thermo Electron Co.) at 25,000 g for 30 minutes. A characteristic dark-colored aqueous suspension was obtained as result of this sonication process.

#### Adsorption of HRP on SWNTs

Sodium phosphate (Sigma, St. Louis, MO), horseradish peroxidase (Worthington, NJ, U.S.A.), and a 10 kDa and 100 kDa dialysis membrane (Spectra-Por, CA, U.S.A.) were used.

#### Attachment of HRP to SWNTs using EDC and NHS

To perform this step 2-(N-morpholino)ethanesulfonic acid (MES), NaCl, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxy-succinimide, 98% (NHS),  $\beta$ -mercaptoethanol (2-Mercaptoethanol, min 98% C<sub>2</sub>H<sub>6</sub>OS), hydroxylamine, sodium phosphate purchased from Sigma (St. Louis, MO), horseradish peroxidase (HRP) from Worthington (NJ, U.S.A.) and a 100 kDa dialysis membrane (Spectra-Por, CA, U.S.A.) were used.

#### Adsorption of annexin V on SWNTs

Sodium phosphate (Sigma, St. Louis, MO), annexin V, and a 10 kDa and 100 kDa dialysis membrane (Spectra-Por, CA, U.S.A.) were used.

#### Attachment of annexin V to SWNTs using EDC and NHS

To perform this step 2-(N-morpholino)ethanesulfonic acid (MES), NaCl, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N-hydroxy-succinimide, 98% (NHS), 2-mercaptoethanol, hydroxylamine, sodium phosphate purchased from Sigma (St. Louis, MO) and a 100 kDa dialysis membrane (Spectra-Por, CA, U.S.A.) were used.

Binding of SWNT-annexin V on plastic-immobilized phosphatidyl serine

Polypropylene microtiter plates were purchased from Corning Inc. (Acto, MA.)

# **CHAPTER III**

## **METHODS**



### Primer design for PCR and construction of annexin V gene

These steps were previously conducted by the graduate student Naveen Palwai.

### Construction of annexin V gene

A linear vector pET-30 Ek/LIC (Novagen, Madison, WI) for annexin V gene constructions was used. A map of this plasmid is illustrated on Figure 27. *E. coli* NovaBlue GigaSingles™ Competent Cells were used for plasmid amplification (Novagen, Madison, WI). The protein expression host was *E. coli* BL21 (DE3), where the resulting plasmid was transformed into BL21 (DE3) competent cells.

### Construction of expression plasmid pET-30 Ek/LIC-annexin V

The expression plasmid pET-30 Ek/LIC-annexin V was constructed by the graduate student Naveen Palwai.

### Cell transformation

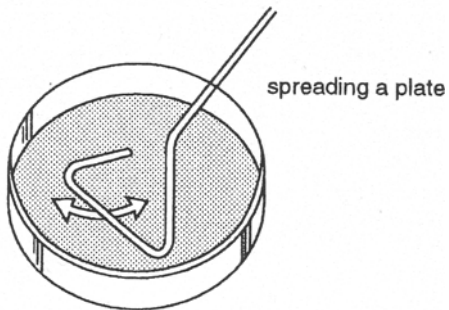
The transformation protocol was followed in order to carry out the transformation (Appendix A.2.1). Two water baths with two different temperatures, 42° C and 100° C, were prepared. The cells (BL 21 DE 3 Cells – pET 30 + 3C + annexin) were removed from the freezer and immediately put in on ice bath (thawing process). The cells were visually examined and carefully agitated to evenly resuspend them. One µl of plasmid (annealing reaction – pET-3C-Annexin) was added to 20 µl cells, stirring carefully to mix. The solution was incubated on ice for 5 minutes, and the tubes were heated exactly 30 seconds in a 42 °C water bath (Water Bath 184, Precision®, Illinois, USA). It is important to not shake the solution. The tubes were immersed in ice for 2 minutes. Eighty µl of room temperature SOC medium was added to each tube (previously, melted on the 100 °C water bath). The tubes were kept on ice until all had received SOC (Appendix

A.1.2). They were incubated at 37 °C while shaking them at 250 rpm for 60 min prior to plating on the selective medium (Figure 49). Plating on the medium containing kanamycin accomplishes selection of transformants. The plasmid has a drug encoding resistance to the kanamycin provided by the kan resistance marker. In order to distribute the solution containing the bacterial cells, a spreader was used. The spreader is made by heating and bending a Pasteur pipette (see Figures 50 and 51). The spreader was sterilized using ethanol, being exposed to a flame. The spreader was carefully cooled by contacting the surface of the agar plate (Fisher Scientific, New Jersey, USA) that does not contain any cells. Afterwards, they were incubated at 37 °C in the incubator one night.

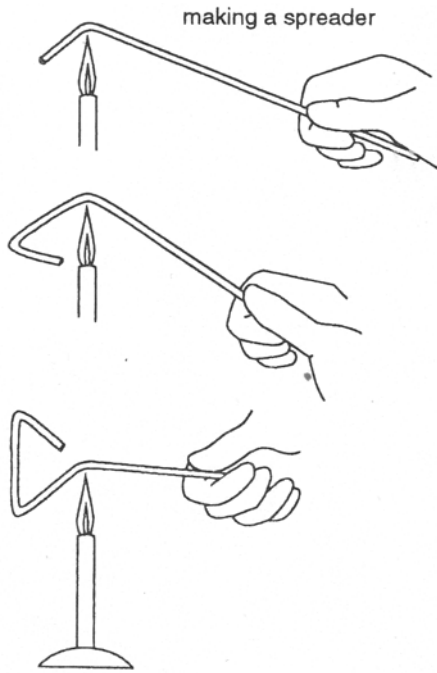
In order to perform the cell expression, the solution was inoculated with a single bacterial colony by touching a sterile inoculating loop (Figure 52) to the colony (placed in the plate), making certain that some of the cells had been transferred to the loop, and then dipping the loop into the liquid medium and shaking it gently (Bloom 1995; Griffiths 2000).



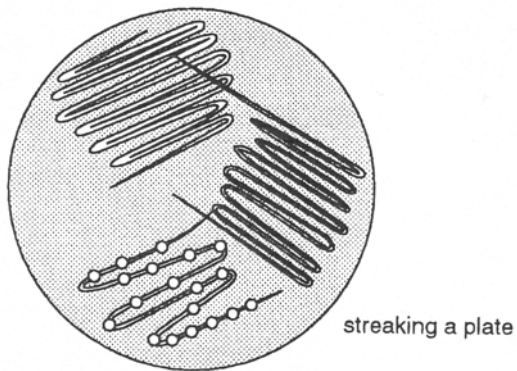
**Figure 49:** Orbital shaker (Queque® Orbital Shaker).



**Figure 50:** Plate illustrating diagram (Ausubel 1987).



**Figure 51:** Illustrating diagram to make a spreader (Ausubel 1987).



**Figure 52:** Plate illustrating diagram (Ausubel 1987).

## Expression of recombinant protein and cell lysis

BL21 (DE3) cells harboring the pET- 30 Ek/LIC plasmid with the annexin V gene (50  $\mu$ l) were cultured in 10 ml of a fresh LB medium containing 35 g kanamycin  $\text{ml}^{-1}$  overnight with constant shaking (250 rpm) at 37 °C in an incubator shaker (Incubator VWR-1540, VWR Scientific Inc., USA) (Figure 49). The overnight cell culture (10 ml) was inoculated into 1 L LB media (Appendix A.1.1), containing the same kanamycin concentration. In order to get a good culture, we used an Erlenmeyer that has at least four times the volume of the culture, providing adequate conditions for cell growing, such as aeration. Cell growth was monitored doing a controlled absorbance measurement (600 nm). A Shimadzu spectrophotometer (Shimadzu UV-2100PC UV-VIS Scanning Spectrophotometer, Japan) was used. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.6 mM to induce the expression of recombinant annexin V when the optical density (600 nm) of the culture reached 0.4-0.6. According to the literature, for a culture grown in a rich medium, each 0.1 optical density unit approximately corresponds to  $1 \times 10^8$  cells per ml. When the absorbance reached this value range, 12 ml of the solution was saved in a small centrifuge tube. After that the small tube (12 ml of solution) was centrifuged (brake = 0, time = 10 mins, speed = 6000 rpm). The pellet was saved and was labeled UNINDUCED. The solution was stirred for 5 hours at 30°C in a Queque Orbital shaker at 250 rpm and centrifuged at 10,000 x g for 10 minutes in a Beckman J2-21 refrigerated centrifuge. After shaking, 10 ml of the solution was saved in a small tube that was called INDUCED.

After that, the pellet was resuspended in 20 ml of sonication buffer (0.05 mM N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01%  $\beta$ -mercaptoethanol and 0.02 M sodium phosphate at pH 7.4). A vortex (Model: G-560, Scientific Industries Inc., Bohemia, NY) was used to mix the solution. After that, the solution was transferred to a small beaker in order to perform the sonication (Sonic Dismembrator, Fisher Scientific, Springfield, NJ) process, causing the cell lysis. The process was performed at 80 % maximum output and at 4 °C for 30 seconds (4.5 watts per ml of lysate) and then allowed to cool for 30 seconds on ice. This

cycle was repeated four times for a total sonication time of 2.5 minutes (Appendix A.1.7). In order to remove cell debris, the cell lysate was centrifuged at 12,000 x g for 30 minutes. The pellet and the supernatant were saved in two different small tubes. The tube containing the pellet was saved in the freezer (-20 °C), and tube containing the supernatant was saved in the refrigerator (because freezing temperatures can promote protein denaturation).

### Protein purification

The fusion proteins expressed using a pET- 30 Ek/LIC vector present a characteristic N-terminal His-tag (His<sub>6</sub>) sequence with an integrated thrombin (Thr) cleavage site, an enterokinase (Ek) cleavage site adjacent to the insert site, and an engineered HRV 3C protease site next to the start of the fusion protein (His<sub>6</sub>/Thr/Ek/3C/Fusion Protein) (Fernandez 1999; Novagen 2006) (Appendix B). The protein purification procedures were executed at 4 °C (Figure 53). The loading of the different solutions was assured by a peristaltic pump (pump speed = 8.00). The entire chromatography system was cleaned and calibrated using 25 ml of wash buffer (20 mM sodium phosphate, 40 mM imidazole, 500 mM NaCl, 0.02 mM pyridoxal phosphate at pH 7.4) to remove unwanted proteins. The system is illustrated in Figure 54. The cell lysate was mixed with 40 mM imidazole (Fisher Scientific) and 500 mM of NaCl was loaded onto a 5 ml HisTrap chromatography column. That buffer was added to the lysate in order to avoid non-specific protein binding. The column was loaded with an elution buffer (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl at pH 7.4) to promote the elution of the His-tagged fusion protein. In order to extract imidazole and NaCl from the protein solution and establish favorable conditions for N-terminal His-tag cleavage, dialysis was performed (Scopes 1982). Fractions containing the purified protein were detected by a UV-Vis spectrometer at 280 nm (Figure 54). Eluted protein was collected onto a dialysis membrane (Spectra-Por, CA, U.S.A.) that was immersed in a dialysis buffer (20 mM sodium phosphate at pH 7.4). The dialysis process was performed overnight at 4 °C using a magnetic stirrer to improve dialysis efficiency.

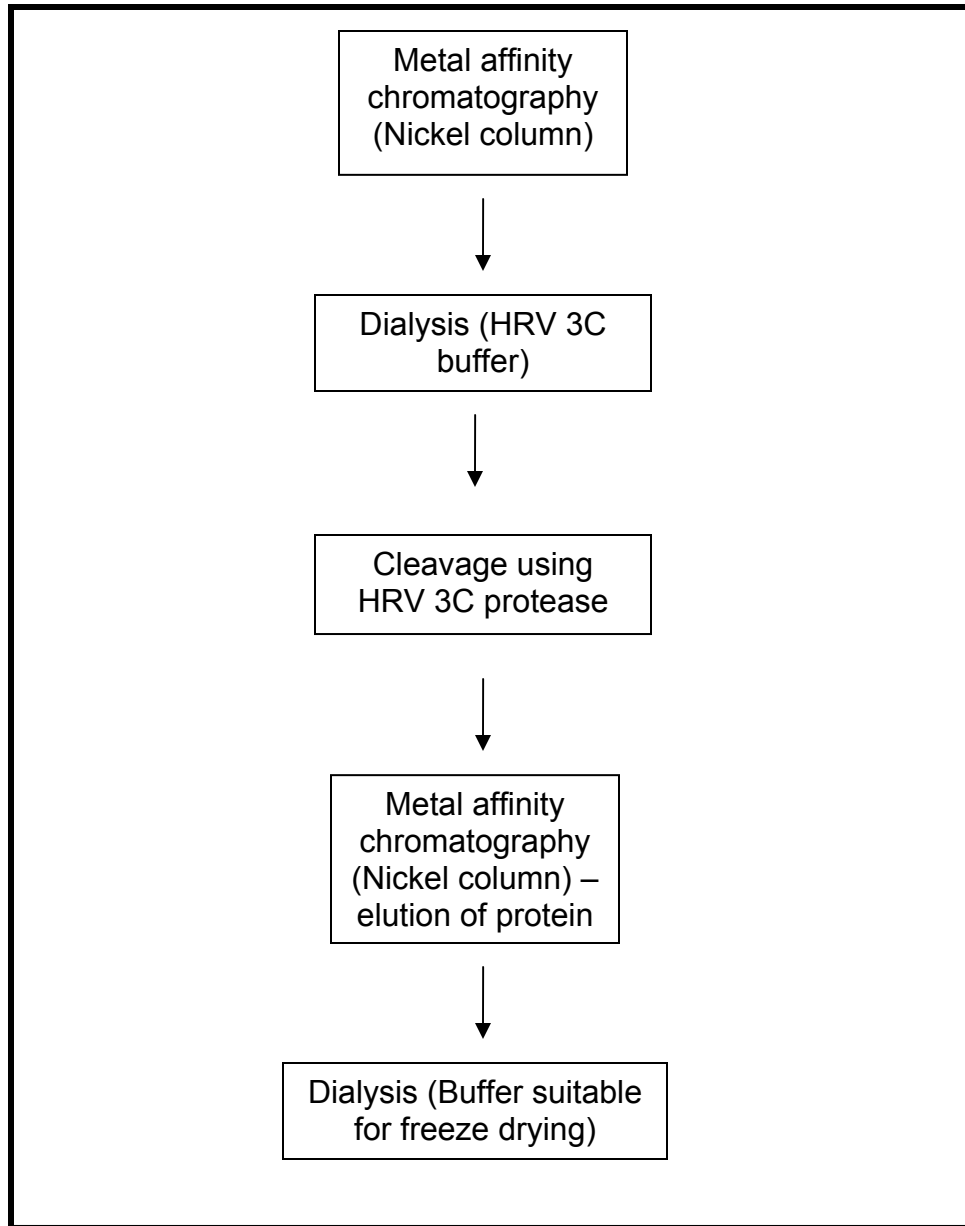
The protein concentration was measured according to the Bradford protein assay protocol (Appendix A.2.2). The Bio-Rad Protein Assay – Dye Reagent Concentrate (# 500-0006, Bio-Rad Laboratories, Hercules, CA) was added at the following volumes:

	Bio-Rad concentrate	Water	Protein Sample
Control tube	200 $\mu$ l	800 $\mu$ l	-
Sample tube	200 $\mu$ l	780 $\mu$ l	20 $\mu$ l

The tubes were gently mixed, and after 10 minutes an absorbance measurement at 595 nm was taken. The value obtained was used to calculate the weight of protein in the solution. After that, HRV 3C protease (Novagen, Madison, WI) was used for the cleavage step, which assures the removal of the N-Terminal His-tag. HRV 3C protease (0.5 Units / mg of protein) and HRV 3C protease cleavage buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) were added to the protein solution. This reaction was performed at 4 °C for 8 hours. Another chromatography, the fusion protein was cleaved. Imidazole (40 mM) and NaCl (500 mM) were mixed with the cleaved protein solution. This solution was loaded onto the 5 ml HisTrap column chromatography. Pure annexin V was eluted using a linear gradient of 0-0.5 M imidazole (Figure 58). The obtained solution was used again for the Bradford protein assay. The pure protein was dialyzed against a dialysis buffer (20 mM sodium phosphate buffer at pH 7.4 containing and 0.1 M NaCl).

The protein was purified directly from the cell lysate using a HisTrap chromatography affinity column that binds to the N-terminal hexahistidine sequence. All procedures were performed in a cold room (4 °C).

After each chromatography, the system was washed in order to avoid system damage. Twenty-five ml of KCl (1 M), 25 ml of NaOH (1 M), 25 ml of water and 25 ml of ethanol (1 M) were loaded into the column. The ethanol remained inside of the system to preserve the column's efficiency.



**Figure 53:** Protein purification steps flow diagram.





**Figure 54:** Overview of the chromatography system.

### SDS PAGE gel preparation

The SDS-PAGE gel (12%) was prepared after the protein purification in order to evaluate protein purity. The gels were prepared according the SDS-PAGE Gel Electrophoresis protocol (Appendix A.2.4). Thirty  $\mu\text{l}$  of buffer (Bio-Rad Laboratories (Hercules, CA)) and 1.5  $\mu\text{l}$  of  $\beta$ -mercaptoethanol ((2-mercaptoethanol, min 98%  $\text{C}_2\text{H}_6\text{OS}$ ) Sigma, St. Louis, MO (1:20) = 1.5  $\mu\text{l}$ )), were added to 30  $\mu\text{l}$  of pellet and mixed, using the vortex. The  $\beta$ -mercaptoethanol was added to avoid protein dimerization. The solution and the marker (*ladder*) (Bio-Rad Prestained SDS-PAGE Standards Broad Range (Catalog 161-0318 Control 310001732), Bio-Rad Laboratories, Hercules, CA) were heated in a water bath (100 °C) for 5 minutes.

The plastic support was introduced inside of the appropriate tank for electrophoresis (SDS-PAGE gel casting stand – Bio-Rad Mini Protean<sup>®</sup> 3Cell, Bio-Rad Laboratories, Hercules, CA). That tank was filled with an electrophoresis buffer (10X buffer i.e. 900 ml of water + 100 ml of buffer) (Bio-Rad 10x Tris / Glycine / SDS Buffer, Bio-Rad Laboratories, Hercules, CA). When the gel was ready, 10  $\mu\text{l}$  of ladder and 15  $\mu\text{l}$  of the solution were loaded to lanes 1 and lanes 2, respectively. Ten  $\mu\text{l}$  of Laemmli Sample buffer (BIO-RAD Laboratories (Hercules, CA) was loaded to the other lanes in order to assure a good migration profile.

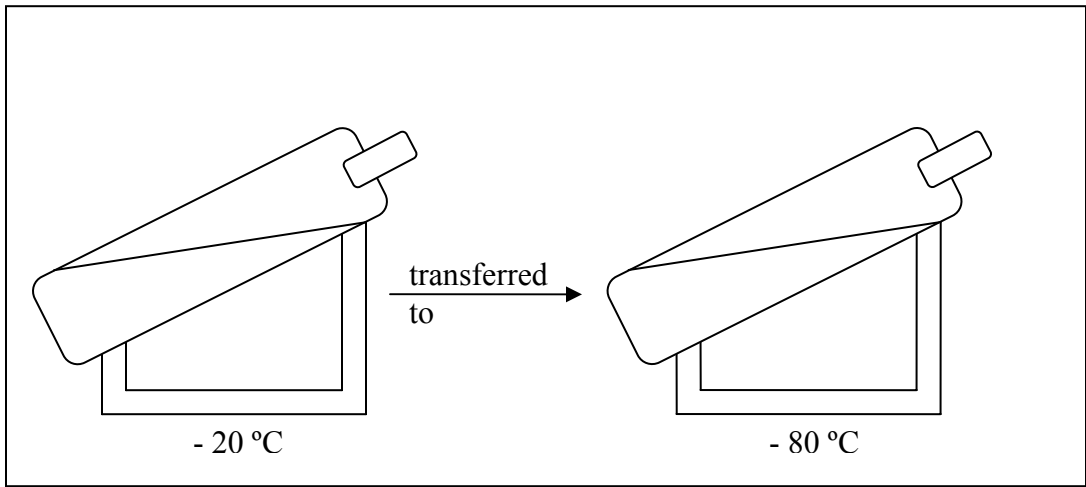
The power source (ISS-enprotech ISS 500, Integrated Separation Systems) was turned on and adjusted to 165 V. The electrophoresis was complete when the bands reached the bottom of the gel. Subsequently, the voltage supply was turned off and the support was removed from the tank.

The gel was stained using a staining solution (Coomassie Brilliant Blue R250 ( $\text{C}_{43}\text{H}_{44}\text{N}_3\text{O}_7\text{S}_2\text{Na}$ )) for 2 hours and after which was destained (45 % (w/v)  $\text{dH}_2\text{O}$ , 45% (w/v) methanol (Fisher Scientific –  $\text{CH}_3\text{OH}$  (New Jersey, USA)), 10 % (v/v) acetic acid). A shaker (Orbit Water Bath Shaker Cat. No. 3535 – Lab-Line Instruments, INC., Illinois, USA) was used for 3 hours. Next, the gel was observed on a light table (Variquest 100 – White Light by Fotodyne – Hearland, WI). Ladder (Bio-Rad Prestained SDS-PAGE

Standards Broad Range, Bio-Rad Laboratories, Hercules, CA) was used as a molecular weight standard.

#### Freeze drying / lyophilization

The freeze drying process was performed according to the freeze drying protocol (Appendix A.2.5), using a Hetosicc Heto FD 2.5 (Heto Lab Equipment). The pure protein solution was introduced in an appropriate freeze drying flask (80 ml) followed by a progressive freezing process at - 80 °C (see the illustrated diagram – Figure 55).



**Figure 55:** Illustrated diagram of the freezing process.

The flask containing the completely frozen solution was introduced into a styrofoam box filled with ice. Once the flask was connected to the system starting the freeze drying process, the pressure began to decrease and was monitored throughout the process. When the pressure reached the lowest value allowed by the protocol, the sample was removed from the ice container, continuing the process at room temperature for approximately 42 hours. To avoid the loss of the sample, through suction caused by the system, filter paper piece was placed on the outlet of the flask (Whatman, Maidstone, England), which acted as a physical barrier while allowing the vapor to escape. When the protein was completely dried, it was saved.

#### Preparation of the SWNT suspension using sodium cholate or low-viscosity sodium carboxymethylcellulose as a dispersion agent

The preparation of the SWNTs suspension was performed using purified and freeze dried CoMoCAT (6,5) and (7,6) single-walled carbon nanotubes (SouthWest Nanotechnologies, Inc.) with diameters of 0.757 and 0.895 nm, respectively. The sample has the majority of nanotubes in the diameter range  $0.8 \pm 0.1$  nm. The dispersants used in this step were sodium cholate (NaC, from Sigma, St. Louis, MO) and low-viscosity sodium carboxymethylcellulose (CMC, from Sigma, St. Louis, MO). The nanotubes and the dispersants were used without a further purification step.

To prepare the nanotube suspensions, SWNTs were added to a 2 wt % NaC or 1 wt % CMC solution and ultrasonicated for 30 minutes with a horn sonic dismembrator (CPX 750, Cole-Parmer) equipped with a microtip of 1/8" in diameter. The resulting suspension was centrifuged in an automatic centrifuge (IEC Multi, Thermo Electron Co.) at  $25,000 \times g$  for 30 minutes. A characteristic dark-colored aqueous suspension was obtained as result of this sonication process (Figure 56).



**Figure 56:** SWNTs suspended in sodium cholate. A characteristic dark-colored aqueous suspension was obtained as result of this sonication process (Palwai 2007).

### Adsorption of horseradish peroxidase on SWNTs

In order to perform the adsorption of annexin V on single-walled carbon nanotubes, an experimental procedure using horseradish peroxidase was primarily used. Sodium phosphate (20 mM) was mixed with the SWNT suspension (7 ml) (3 mg 8L SWNT in 2% sodium cholate). Twenty mg of HRP was added to that solution. The solution was dialyzed against a 2 L sodium phosphate buffer (20 mM) (12 h, pH 7.4) using a 10 kDa dialysis membrane. After that time period, the solution was transferred to a 100 kDa dialysis membrane, which was dialyzed against a 2 L sodium phosphate buffer (20 mM) (pH 7.4). The dialysis was performed during 8 hours, with a buffer replacement after the first 4 hours. The solution was centrifuged at 29,600 x g for 1h. The supernatant obtained was saved. The protein in the solution was quantified performing the Bradford protein assay (Appendix A.2.2).

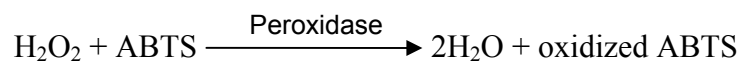
The Bio-Rad Protein Assay – Dye Reagent Concentrate (# 500-0006, Bio-Rad Laboratories, Hercules, CA) was added according to the following volumes:

	Bio-Rad concentrate	Water	Protein Sample
Control tube	200 $\mu$ l	800 $\mu$ l	-
Sample tube	200 $\mu$ l	780 $\mu$ l	20 $\mu$ l

The tubes were gently mixed waiting 10 minutes prior to the absorbance measurement at 595 nm. The obtained value was used to calculate the protein concentration in our solution. The absorbance of the pre-starting SWNT, pre-centrifuging SWNT-HRP complex and post-centrifuging SWNT-HRP complex was measured at 800 nm.

A UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-900 nm, while a Bruker Equinox 55 FTIR was used for the NIR range, 850-1,350 nm. A UV-Vis-NIR spectrum was obtained, and it is presented in the Results section (Figure 61).

The HRP activity was quantified by performing an enzyme activity assay (Appendix A.2.3). Basically, the H<sub>2</sub>O<sub>2</sub> is the initiator of the reaction reacting with the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) that is the substrate. When enzymes are present in solution those two reagents are converted to water and oxidized ABTS.



When in the presence of an enzyme with some activity, the reaction causes an increase of the absorbance, with a characteristic absorbance stabilization after the first minute, since the maximum linear rate occurs within the first minute of the reaction (Appendix A.2.3). This assay is based on the continuous spectrophotometric rate determination (25 °C, pH = 5.0, A<sub>595nm</sub>, light path = 1 cm) method. The ΔA<sub>405nm</sub>/minute is calculated using the maximum linear rate for the test and blank samples. According to the definition, one unit will oxidize 1.0 μmole of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) per minute at pH 5.0 at 25 °C. The obtained values are presented in the Results section (Figure 60).

The measurement was repeated after 5 days, and the solution during that period remained at room temperature. The activity and protein concentration was also measured in the dialysate, in order to investigate the possibility of some remaining protein in that solution.

The initial solution (SWNT dispersed into sodium cholate) and the final solution (SWNT-HRP complex) were analyzed by atomic force microscopy (AFM) (Figure 62).

#### Attachment of HRP to SWNTs using EDC and NHS

The attachment of HRP to SWNTs using EDC and NHS requires an initial 8 h dialysis (0.5 M NaCl), that removes the excess and unbounded CMC in the initial suspension (SWNT-CMC). The buffer was replaced after 4 h from the start of dialysis.

The procedure for two-step coupling of SWNT-CMC (3 mg 8L SWNT in 1% low viscosity CMC) and HRP using EDC and NHS is described here. The activation buffer



(0.1 M MES (2-(N-morpholino)ethanesulfonic acid), 0.5 M NaCl, pH 6.0) was mixed with the 7 ml SWNT-CMC solution (estimated CMC concentration = 0.345 mg/ml).

The EDC and NHS were equilibrated to room temperature before opening the bottles. EDC (2.8mg ~ 2 mM) and 4.2 mg of NHS were added to 7 ml of SWNT-CMC solution with the activation buffer reacting for 15 minutes at room temperature. In order to quench the EDC, 9.8  $\mu$ l of 2-mercaptoethanol was added to a final concentration of 20 mM. The protein (HRP) was added to the activated CMC at an equal weight ratio with CMC. The reaction took place at room temperature for 2 hours. To quench the reaction, hydroxylamine was added to a final concentration of 10 mM. This method hydrolyzes nonreacted NHS present on CMC and results in the regeneration of the original carboxyls. Other quenching methods involve adding 20-50 mM Tris, lysine, glycine or ethanolamine; however, these primary amine containing compounds modify carboxyls on CMC (Pierce Catalog, 2006).

In order to remove any excess reagent, a dialysis was performed using a 100 kDa dialysis membrane immersed in a 2 L sodium phosphate buffer (20 mM) (pH 7.4). The buffer was replaced after 4 h from the start of dialysis, which lasted for a total of 8 h. The final volume inside the dialysis membrane was measured. A centrifugation was performed at 29,600 x g for 1 h, in order to isolate the SWNT-CMC fraction bound to annexin V. The supernatant was retained. Three samples (100  $\mu$ l) were saved to be analyzed by AFM, UV-Vis-NIR absorption spectra and Raman spectra. An initial SWNT-CMC sample was also saved. The protein concentration in the final suspension (obtained after centrifugation) and in the dialysate was measured using the Bradford protein assay.

The HRP activity was quantified by performing an enzyme activity assay (Appendix A.2.3). The measurement was repeated after 5 days, with the solution holded during that period at room temperature. The activity and protein concentration was also measured in the dialysate, in order to investigate the possibility of some remaining protein on that solution.

### Adsorption of annexin V on SWNTs

In order to evaluate the adsorption of annexin V on SWNTs, sodium phosphate (20 mM) was mixed with the SWNT suspension (1.75 ml) (3mg 8L SWNT in 2% sodium cholate). Five mg of previously prepared annexin V was added to that solution. The solution was dialyzed against a 500 ml sodium phosphate buffer (20 mM) (12 h, pH 7.4) using a 10 kDa dialysis membrane. After that time period, the solution was transferred to a 100 kDa dialysis membrane, which was dialyzed against a 2 L sodium phosphate buffer (20mM) (pH 7.4). The dialysis was performed during 8 hours, with a buffer replacement after the first 4 hours. The solution was centrifuged at 29,600 x g for 1h. The supernatant obtained was saved. The protein in the solution was quantified performing the Bradford protein assay (Appendix A.2.2).

The BIO-RAD Protein Assay – Dye Reagent Concentrate (# 500-0006, Bio-Rad Laboratories, Hercules, CA) was added according to the following volumes:

	Bio-Rad concentrate	Water	Protein Sample
Control tube	200 µl	800 µl	-
Sample tube	200 µl	780 µl	20 µl

The tubes were gently mixed waiting 10 minutes prior to the absorbance measurement at 595 nm. The obtained value was used to calculate the protein concentration in our solution. The absorbance of the pre-starting SWNT, pre-centrifuging SWNT-HRP complex and post-centrifuging SWNT-HRP complex was measured at 800 nm.

### Attachment of annexin V to SWNTs using EDC and NHS

The adsorption of annexin V on SWNTs using EDC and NHS requires an initial 8 h dialysis (0.5 M NaCl), that removes the excess and unbounded CMC in the initial suspension (SWNT-CMC). The buffer was replaced after 4 h from the start of dialysis.

The procedure for two-step coupling of SWNT-CMC (3mg 8L SWNT in 1% low viscosity CMC) and annexin V using EDC and NHS is described here. The activation buffer (0.1 M MES (2-(N-morpholino)ethanesulfonic acid), 0.5 M NaCl, pH 6.0) was mixed with the 7 ml SWNT-CMC solution (estimated CMC concentration = 0.345 mg/ml).

The EDC and NHS were equilibrated to room temperature before opening the bottles. EDC (2.8 mg ~ 2 mM) and 4.2 mg of NHS were added to 7 ml of SWNT-CMC solution with the activation buffer reacting for 15 minutes at room temperature. In order to quench the EDC, 9.8  $\mu$ l of 2-mercaptoethanol was added to a final concentration of 20 mM. The protein (HRP) was added to the activated CMC at an equal weight ratio with CMC. The reaction took place at room temperature for 2 hours. To quench the reaction, hydroxylamine was added to a final concentration of 10 mM. This method hydrolyzes nonreacted NHS present on CMC and results in the regeneration of the original carboxyls. Other quenching methods involve adding 20-50 mM Tris, lysine, glycine or ethanolamine; however, these primary amine containing compounds modify carboxyls on CMC (Pierce Catalog, 2006).

In order to remove any excess reagent a dialysis was performed using a 100 kDa dialysis membrane immersed in a 2 L sodium phosphate buffer (20 mM) (pH 7.4). The buffer was replaced after 4h from the start of dialysis, which lasted for a total of 8 h. The final volume inside the dialysis membrane was measured. A centrifugation was performed at 29,600 x g for 1 h, in order to isolate the SWNT-CMC fraction bound to annexin V. The supernatant was retained. Three samples (100  $\mu$ l) were saved to be analyzed by AFM, UV-Vis-NIR absorption spectra and Raman spectra. An initial SWNT-CMC sample was also saved. The protein concentration in the final suspension (obtained after centrifugation) and in the dialysate was measured using the Bradford protein assay.

Binding of SWNT-annexin V on plastic-immobilized phosphatidyl serine

In order to study the binding of the SWNT-annexin V complex to the plastic-immobilized phosphatidylserine a solution with phosphatidyl serine and chloroform (50  $\mu\text{g/ml}$ ) was prepared. To each well of a 96-well polypropylene microtiter plates, 100  $\mu\text{l}$  of that solution was added in triplicate. A laminar flow hood was used in order to evaporate all the chloroform. The plate was washed with a phosphate buffer saline (PBS) solution (10% fetal bovine serum and 2 mM calcium chloride) for 2 h at room temperature in the laminar flow hood. The SWNT-annexin V solution was diluted to 6.7 nM (protein) using a wash buffer. The complex SWNT-annexin V was diluted with a wash buffer (6.7 nM) to obtain the final concentration of 6.7 pM. The SWNT suspended in sodium cholate (original suspension) solution was our control solution. The solution was incubated in each well (100  $\mu\text{l}$ ) of the plate for 2 h. The following step was washing the plates with the wash buffer (2x). The samples were analyzed using Raman spectroscopy in order to detect the binding annexin V-PS.

# **CHAPTER IV**

## **RESULTS AND DISCUSSION**

During the different experiment stages, it is very important to be precise and accurate in order to minimize possible experimental errors. An attempt was made to optimize the experimental protocols.

### Cloning, overexpression and purification of annexin V

In bacterial cell growth it is extremely important to provide ideal conditions when trying to maximize the growth without any possible contaminant. In few initial stages of this work, cell growth was not observed. To attempt to determine the reason for this, changes of some details of the original protocol were made.

Overexpression of annexin V was attempted using an initial cell culture of 50 ml incubated at 37 °C overnight. However, because of the failure of this protocol, it was necessary to reduce 5x the volume of the initial cell culture.

Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture with a concentration of 0.6 mM instead of 1 mM to promote slow and controlled cell growth. The cell induction was carefully controlled, at a constant temperature of 30°C with a constant shaking. This is the optimized temperature to perform this step that has a temperature-sensitive character. In this section, a brief explanation is presented about some problems encountered during the different stages of the experiment. The resolution of these problems brought about the optimization of our protocols and methods.

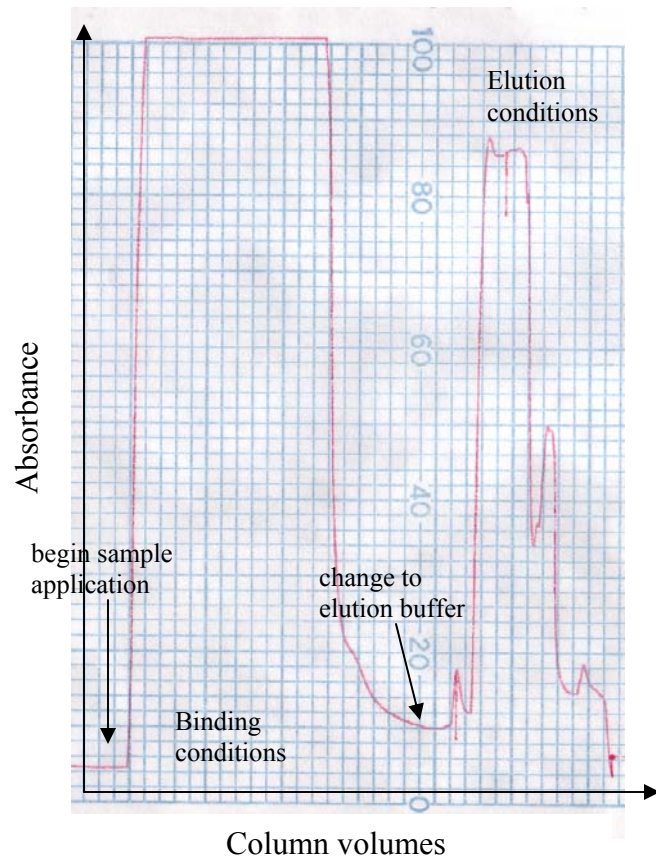
Five hours after induction, the cell pellets were lysed by a sonication process, and the cell debris was removed by centrifugation. During the protein purification, a new column chromatography was used in order to assure the best possible purification yield. It is important to note that the purification and other steps using the protein annexin V should be performed continuously if possible or with short interruption periods, in order to try to avoid protein denaturation and consequently its loss of function. Some processes are performed at a cold temperature (4 °C) and the storage was performed at 4 °C or -20 °C.

When the protein purification step is performed, it requires the use of a chromatography column. The pumping of different buffered solutions into the column

allows for the purification of the protein. An important factor related with the pumping is the processing rate that directly influences the final yield.

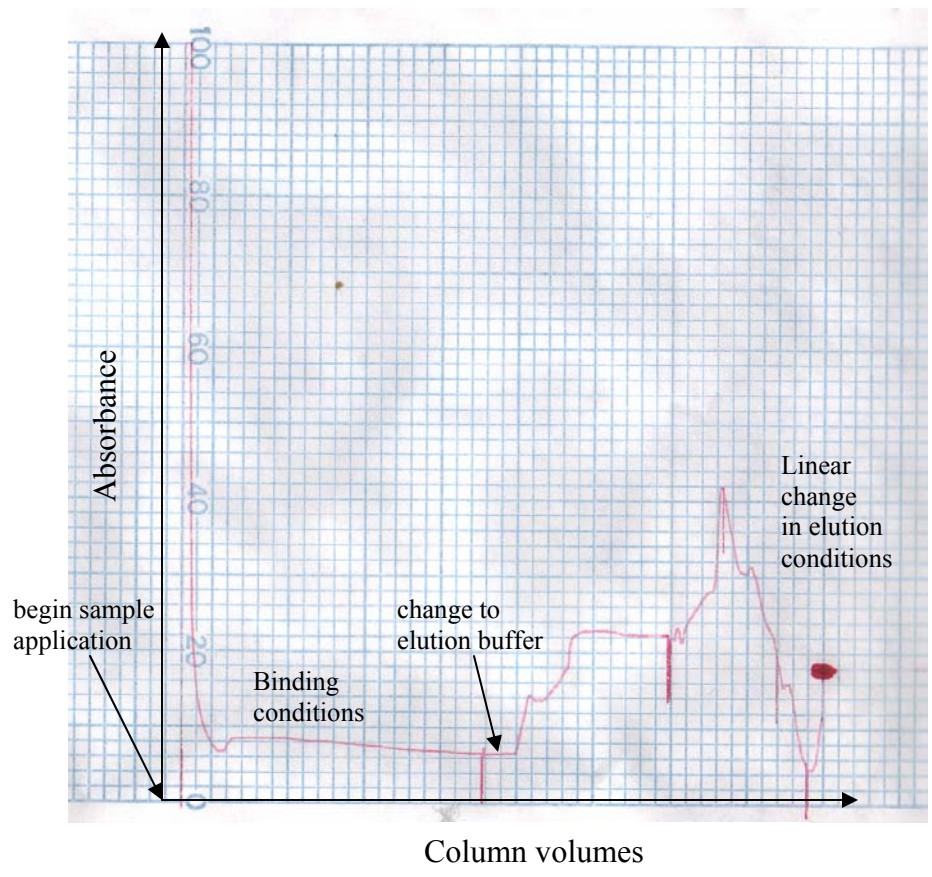
The loading of the sample into the column causes an increase of the absorbance measured at 280 nm that indicates the adsorption of the sample and elution of unbound material. Then, the unbound material is washed away and the eluting buffer is loaded into the column, allowing the elution of bound proteins – step elution (Figures 40, 41a and 57).

The use of a gradient elution in the chromatography system allows the movement of strongly retained components of the mixture to proceed at a faster rate. Starting with a low concentration of the organic compound (e.g. imidazole) in the eluent allows the least retained components to be separated. Strongly retained components will stay on the adsorbent surface at the top of the column, or will move very slowly. When the concentration of the organic component starts to increase, then strongly retained components will move faster, caused by the steady increase of the competition for the adsorption sites. The efficiency of the column is maximized by using a gradient elution (Figures 41b, 42 and 58).



**Figure 57:** Step elution using affinity chromatography.





**Figure 58:** Gradient elution of a (His)<sub>6</sub> fusion annexin V using affinity chromatography.

During the purification, the protein concentration was measured using the Bradford protein assay (Appendix A.2.2). The weight of protein in our solution (22 mg of protein) was determined. With this value, it was possible to calculate the necessary amount of HRV 3C Protease to add to our sample (110  $\mu$ l).

$$\text{HRV 3C Protease Amount } (\mu\text{l}) = \left[ \frac{\left( \frac{22000 \mu\text{g} \cdot 1U}{100 \mu\text{g}} \right) \cdot 1000 \mu\text{l}}{2000U} \right] = 110 \mu\text{l}$$

HRV 3C Protease Activity = 2000 U/ml (Appendix A.1.8)

Since 1 unit will cleave approximately 100  $\mu$ g His-tag fusion control (Appendix A.1.8), it was necessary to use 220 units in our specific sample (22 mg). The cleavage step was performed, adding 110  $\mu$ l of HRV 3C Protease to a 27 ml sample.

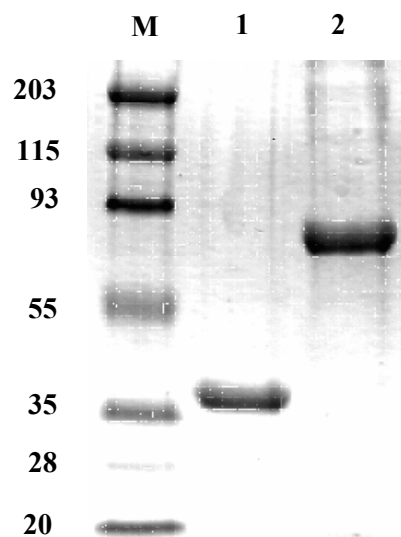
The final protein concentration was measured, as described by Bradford (Bradford 1976), with bovine serum albumin (Fraction V, Minimum 96%) as reference (Bradford protein assay (Appendix A.2.2)), and the value of 145 mg/L was determined (Table 7).

**Table 7:** Annexin V properties after purification and HRP properties (Welinder 1979; Catalog 2006).

<b>Protein</b>	<b>Amino Acids</b>	<b>Theoretical Molecular Weight (kDa)</b>	<b>Protein Concentration (mg/L)</b>	<b>Specific Activity (U/mg)</b>
Annexin V	321	35.92	145	0
Horseradish Peroxidase	308	40	239	167

## SDS-PAGE

The proteins in both samples were separated by SDS-PAGE gel electrophoresis followed by Coomassie Blue staining in order to quantify the purity of the protein. Figure 59 shows the purity of the protein annexin V, obtained after the expression, purification and freeze drying processes. The markers indicate that the purified annexin V is close to its theoretical molecular size (36 kDa).



**Figure 59:** Coomassie blue 12 % sodium dodecyl sulfate-polyacrylamide gel of the various purification steps. Lane M, Marker ladder (Bio-Rad, Hercules, CA) with the indicated molecular masses (10  $\mu$ l). Lane 1, annexin V prepared at 1 mg/ml after the freeze drying step; Lane 2, bovine serum albumin prepared at 1 mg/ml.

### Single walled carbon nanotubes

The centrifugation of the solution containing the nanotubes in suspension with sodium cholate gave a solution with nanotubes totally dispersed (individually). Sodium cholate removal was performed using a 10 kDa dialysis membrane, retaining the annexin V-SWNT complex.

### Adsorption of HRP on SWNTs

To conserve annexin V, adsorption of the model protein horseradish peroxidase (HRP) directly on SWNTs was attempted first using an existing protocol. This protocol was optimized in order to maximize the yield of protein adsorption on SWNTs. Enzyme activity and protein concentration results for HRP adsorbed on SWNT are given in Table 8. The enzyme activity / enzyme activity of native protein (0.92) is similar to that obtained in the previous experiment (Palwai 2007). Measurements taken after holding the SWNT-HRP suspension for 5 days at room temperature indicate a complete loss of enzymatic activity. The finding of no enzyme activity or protein in the final 2 L dialysis solution indicates that HRP was all adsorbed on the SWNTs. The sample was analyzed by AFM, making it possible to visualize the HRP attached to the nanotubes (Palwai 2007).

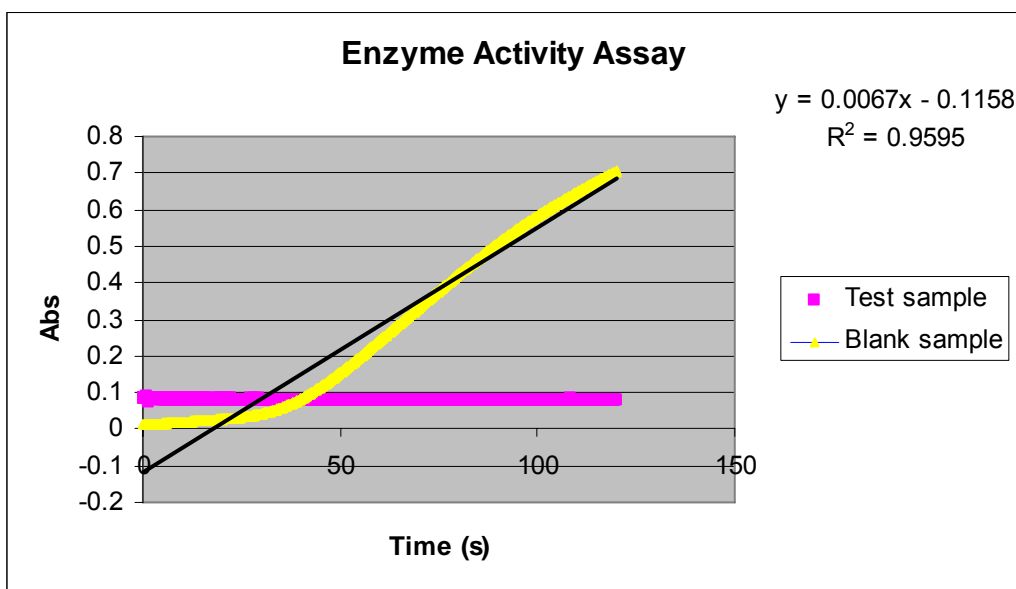
Performing the Bradford protein assay (Appendix A.2.2), a value of 266 mg/L was obtained. The enzyme activity assay was performed according to the respective protocol (Appendix A.2.3). The results are presented in the Table 8 and in the Figure 60. The enzyme activity for the SWNT-protein suspension after centrifugation was 189 U/mg and the SWNT concentration was 153 mg/L that corresponds to a 78.9 % recovery (Table 9). The ratio between protein weight and SWNT weight (protein loading) was equal to 1.7.

The initial suspension (SWNT dispersed in 2 wt % sodium cholate) and the SWNT-protein suspensions before and after centrifuging were analyzed by UV-Vis-NIR spectroscopy. The nanotube concentration was determined from the absorbance at 800

nm that corresponds to a valley in the absorbance spectrum (Figure 61) where the absorbance is minimally influenced by the specific  $S_{11}$  transitions of the SWNT or by the adsorbed protein. From a direct analysis of the UV-Vis-NIR absorbance spectrum, it is possible to note that in the 300-400 nm wavelength range of the spectra, the absorbance was significantly greater for both of the samples with SWNT-protein than for SWNTs alone, and the SWNT-protein after centrifugation had significantly higher absorbance in this same range than the SWNT-protein before centrifugation. This latter difference indicates that the SWNT-protein before centrifugation contained SWNTs with varying amounts of protein adsorbed per SWNT, and after centrifugation the SWNTs with lower amounts of protein adsorbed were removed.

A strong absorption band at 980 nm is present, which is a typical band for CoMoCAT samples and attributed to the  $S_{11}$  transition of (6,5) nanotubes (Figure 17). The intensities of the peaks were similar for the pristine SWNTs and the SWNT-protein pre-centrifugation samples. However, there is a slight, but consistent red shift in the SWNT-protein samples that can be explained as a stronger SWNT-protein interaction than with the surfactant.

The atomic force microscopy images present strong evidences that corroborate the success of this experiment. The arrows indicate the adsorption of sodium cholate (Figure 62 a) and HRP (Figure 62 b). The height of the aggregates of HRP in the final suspension (3.8-6.0 nm) is considerably higher than those of sodium cholate (1.0-1.5 nm), and this fact is consistent with the size of similar proteins that have about the same molecular weight as HRP. For example,  $\beta$ -lactoglobulin has a 35 kDa molecular weight and a diameter of 5.4 nm (Palwai 2007).



**Figure 60:** Enzyme activity assay.



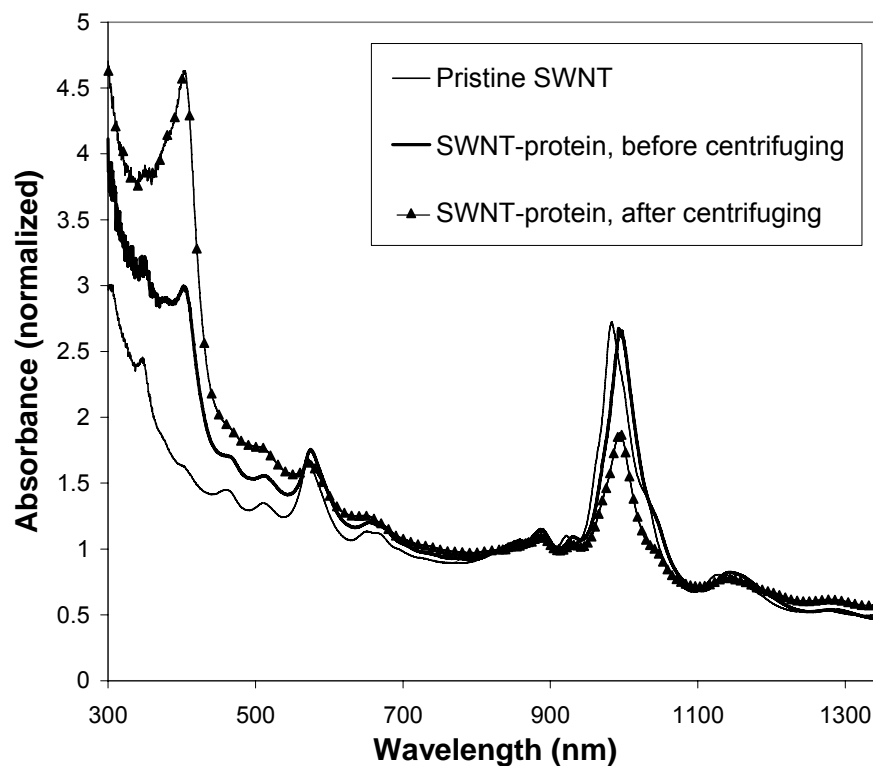
**Table 8:** Enzyme activity and protein concentration results for HRP.

<b>Sample</b>	<b>Enzyme activity, U/mg protein</b>	<b>Protein concentration, (mg/L)</b>	<b>Enzyme activity/ enzyme activity of native protein</b>	<b>SWNT Concentration, (mg/L)</b>	<b><u>Protein Weight</u>, SWNT Weight (mg/mg)</b>
SWNT-protein suspension after centrifugation					
Day 0	189	266	0.92	153	1.7
Day 5, held at room temperature and centrifuged again	0	16	0	–	–
Final dialysis solution (2 L) using 100 kDa membrane	0	0	–	–	–

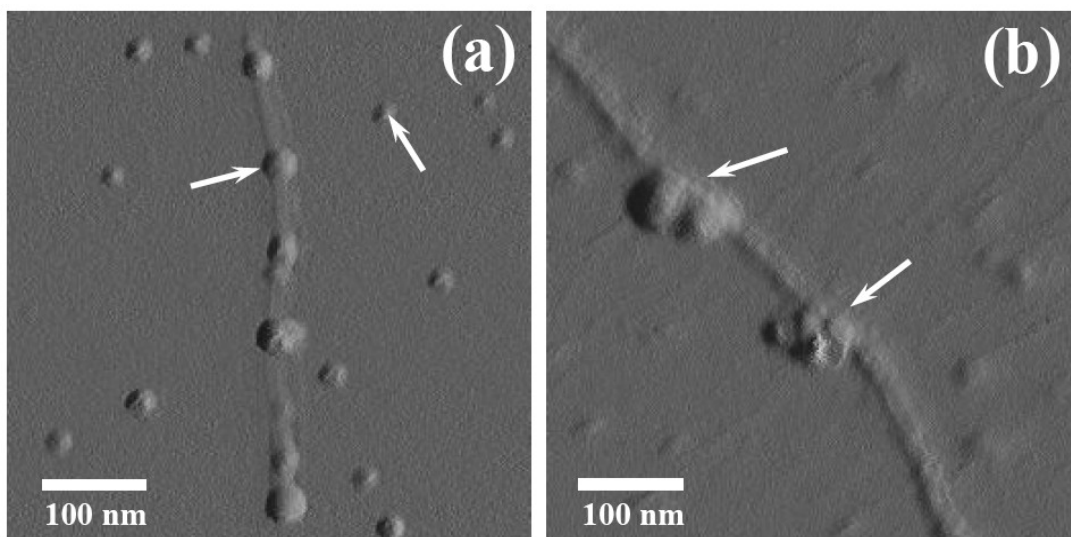
**Table 9:** SWNT concentrations, as measured by the absorbance at 800 nm.

Compound	SWNT concentration, mg/L	Yield, %
Pristine SWNTs	194	100.0
SWNT-protein, pre-centrifugation	174	89.6
SWNT-protein, post-centrifugation*	153	78.9

\*From Table 8 data, protein weight/ SWNT weight = 1700  $\mu\text{g}/\text{mg}$  (= 42.5 nmol protein/mg SWNTs)



**Figure 61:** UV-Vis-NIR absorbance spectrum (normalized). A UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-900 nm while a Bruker Equinox 55 FTIR was used for the NIR range, 850-1,350 nm.



**Figure 62:** AFM images showing SWNT with sodium cholate and HRP protein. (a) SWNT in sodium cholate after sonication and centrifugation. Arrows indicate solid sodium cholate with a height of 1.0-1.5 nm (does not include 0.8 nm SWNT height). (b) SWNT-protein after dialysis and centrifugation. Arrows indicate protein associated with SWNT. The height is 3.8-6.0 nm (does not include 0.8 nm SWNT height).

### Adsorption of annexin V on SWNTs

In this study, we tried to perform the adsorption of annexin V on SWNTs, using the same protocol used for the HRP protein. However, during the experiment and after the final dialysis, the SWNT completely precipitated (formation of nanotube aggregates). After the centrifugation, the soluble fraction was completely clear, showing the absence of nanotubes associated with the annexin V in suspension.

With this important result, new methodologies were searched in order to make it possible to attach the protein to SWNTs. Pegylation was a possible method evaluated, with PEG used to suspend the nanotubes by adsorbing to them and then covalently coupling PEG and annexin V. However, it was not possible to find a PEG that could be purchased for this purpose. Since Dr. Resasco's group had already shown that SWNTs could be suspended using carboxymethylcellulose (CMC), the coupling of annexin V to the CMC-SWNT complex was evaluated.

That result led to the development of a new protocol, using a one-step EDC reaction with carboxyl and amine-containing molecules (Figure 48).

### Attachment of HRP to CMC-SWNTs using EDC and NHS

The attachment of the protein HRP for the CMC-SWNTs complex was evaluated using EDC and NHS, according to the protocol presented in the methods section. The preliminary results for this reaction are presented on Table 11.

**Table 10:** Volumes in the global experiment.

Initial volume (ml) of the solution containing SWNT-CMC	6.5
Volume (ml) of the solution after the 2 dialysis (8h) with 2L of water	20
Final volume (ml) after the centrifugation	20

According to Table 10, it is possible to quantify an increasing volume of approximately 3 times. This result is explained by the fact that we did the first two dialysis steps using water, leading to osmosis into the dialysis bag. Osmosis contributes to the dilution of the dialysate. In the following studies, a 0.5 M sodium chloride solution was used instead of water in the first two dialysis steps, in order to remove the excess of CMC (unbound).

Performing the Bradford protein assay (Appendix A.2.3), a value of 239 mg/L was obtained. The enzyme activity assay was carried out according to the respective protocol (Appendix A.2.3). The results are presented in the Table 11 and in Figures 63, 64 and 65. The enzyme activity for the SWNT-CMC-protein suspension after centrifugation was 167 U/mg (Table 11). The ratio between the enzyme activity and the enzyme activity of the native protein was equal to 81 %. The ratio between protein weight and SWNT weight (protein loading) was equal to 3.0. The conjugation of SWNT-CMC with HRP using EDC was successful, with an increase of 76 % of protein loading compared to the adsorption experiment. No enzyme activity or protein was found in the final dialysis solution (2 L), which indicates that unbound annexin V had been removed by the dialysis.

The CMC-HRP-SWNT complex forms a stable suspension after dialysis and centrifugation. The concentration of SWNT in the initial suspension without protein (1% low viscosity CMC) was 320 mg/L (Table 12). The concentration of SWNT in the protein-CMC-SWNT suspension after dialysis and before centrifugation was 290 mg/L. The concentration of protein-CMC-SWNT suspension after dialysis and centrifugation was 81 mg/L that corresponds to a 53 % recovery (Table 12). These data show that there was some loss of activity compared to the native enzyme (enzyme activity/enzyme activity of native protein = 0.81). However, it is encouraging that the protein was 36 % higher than when HRP was attached by adsorption.

The UV-Vis-NIR spectrum for the three samples during this run (initial, pre-centrifugation, and post-centrifugation) is shown in Figure 66. The plot of absorbance at 800 nm versus SWNT concentration is shown in Figure 67.

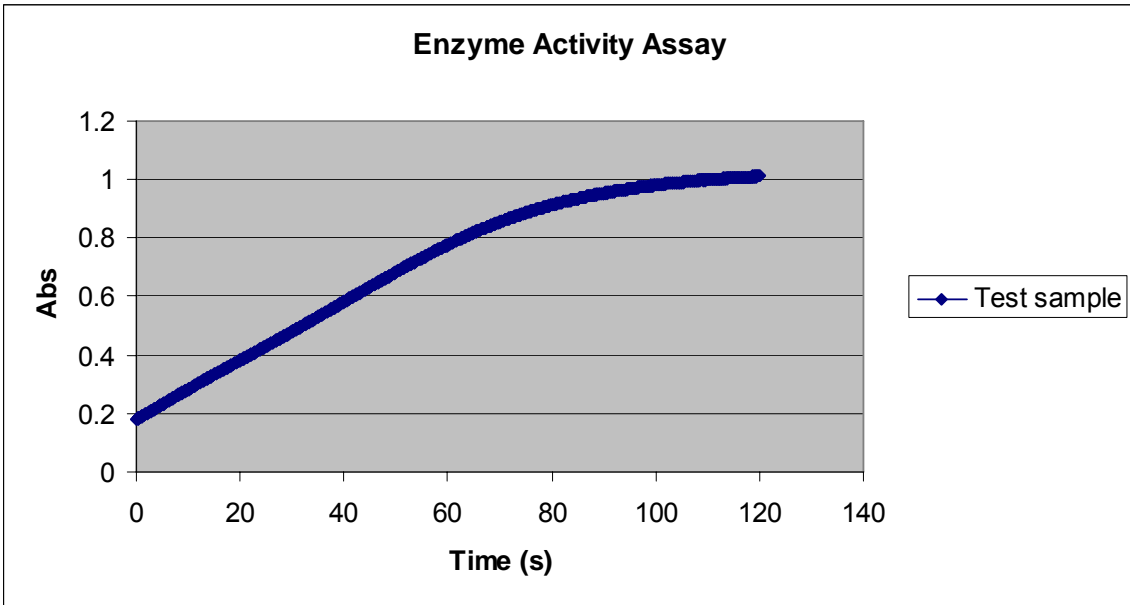


Figure 63: HRP activity assay.

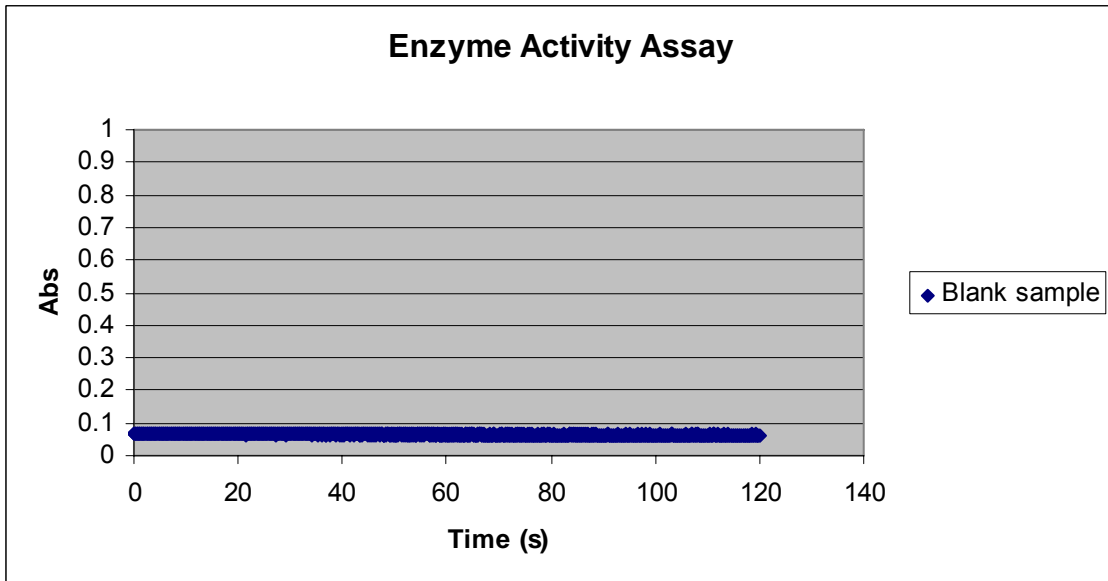
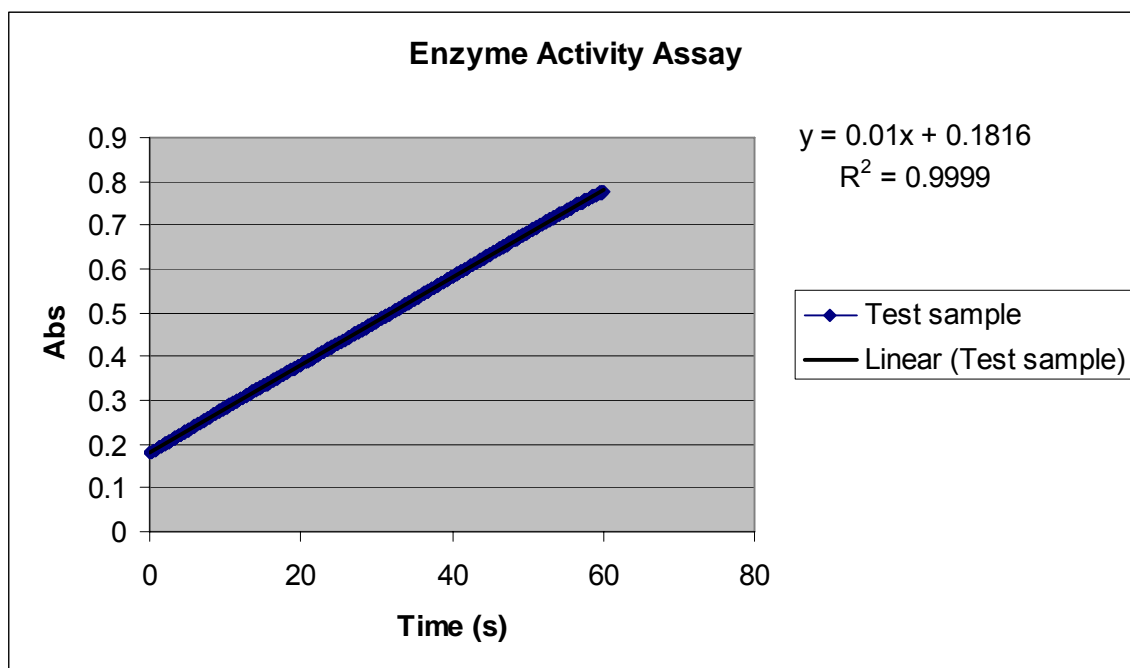


Figure 64: HRP activity assay.



**Figure 65:** Enzyme activity assay.



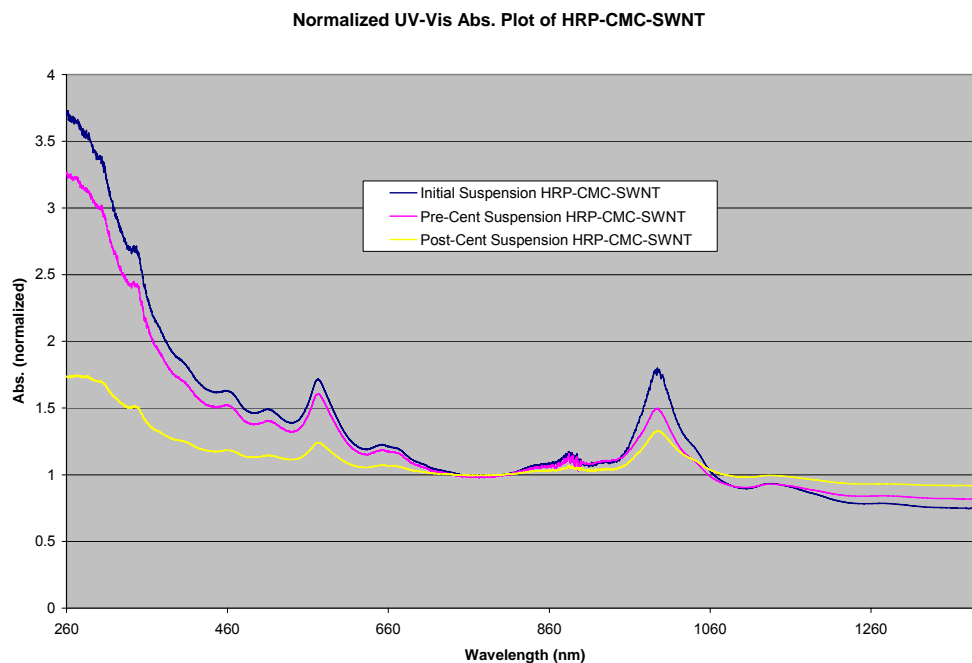
**Table 11:** Enzyme activity and protein concentration results.

<b>Sample</b>	<b>Enzyme activity, U/mg protein</b>	<b>Protein concentration, mg/L</b>	<b>Enzyme activity/enzyme activity of native protein</b>	<b>SWNT Concentration, mg/L</b>	<b><u>Protein Weight</u>, mg/mg SWNT Weight</b>
SWNT-CMC-HRP suspension after centrifugation	167	239	0.81	81	3.0
Final dialysis solution (2 L) using 100 kDa membrane	0	0	0	–	–

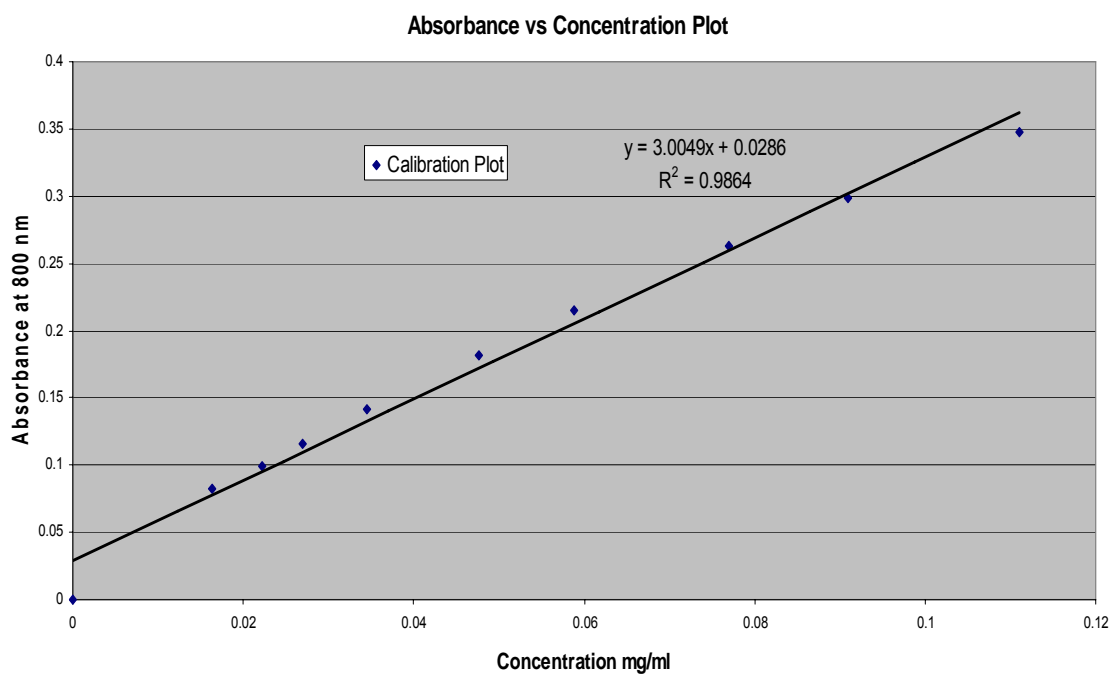
**Table 12:** SWNT concentrations, as measured by the absorbance at 800 nm.

<b>Compound</b>	<b>SWNT concentration, mg/L</b>	<b>Yield, %</b>
Pristine SWNTs	320	100.0
SWNT-protein, pre-centrifugation	290	135.9
SWNT-protein, post-centrifugation*	81	52.7

\*From Table 11 data, protein weight/ SWNT weight = 3000  $\mu\text{g}/\text{mg}$  (= 74.9 nmol protein/mg SWNTs)



**Figure 66:** UV-Vis absorbance spectrum. An UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-1400 nm.



**Figure 67:** Absorbance at 800 nm vs. SWNT concentration plot.

### Attachment of annexin V to SWNT-CMC using EDC and NHS

The attachment of annexin V to SWNT-CMC using EDC and NHS was performed using EDC and NHS, according to the protocol presented in the methods section. The results for this reaction are presented on Table 14.

**Table 13:** Volumes in the global experiment.

Initial volume (ml) of the solution containing SWNT-CMC	6
Volume (ml) of the solution after the 2 dialysis (8h) with NaCl	6
Volume (ml) of the solution after the conjugation reaction using EDC and NHS	12.5
Final volume (ml) after the centrifugation	12.4

According to Table 13, it is possible to quantify an increasing volume of approximately 2 times. This result is explained by the fact that we use a base to adjust the pH of the reaction using EDC and NHS.

Performing the Bradford protein assay (Appendix A.2.3), a value of 74 mg/l was obtained. The ratio between protein weight and SWNT weight (protein loading) was equal to 5.1. The conjugation of SWNT-CMC with annexin V was successful, with an increase of 70 % of protein loading compared to the same experiment using HRP.

The CMC-Annexin V-SWNT complex forms a stable suspension after dialysis and centrifugation. The concentration of SWNT in the initial suspension without protein (1% low viscosity CMC) was 26.6 mg/L (Table 15). The concentration of SWNT in the protein-CMC-SWNT suspension after dialysis and before centrifugation was 25.6 mg/L. The concentration of protein-CMC-SWNT suspension after dialysis and centrifugation was 14.6 mg/L that corresponds to a 113.4% recovery (Table 15).

The UV-Vis-NIR spectrum for the three samples during this run (initial, pre-centrifugation, and post-centrifugation) is shown in Figure 68.

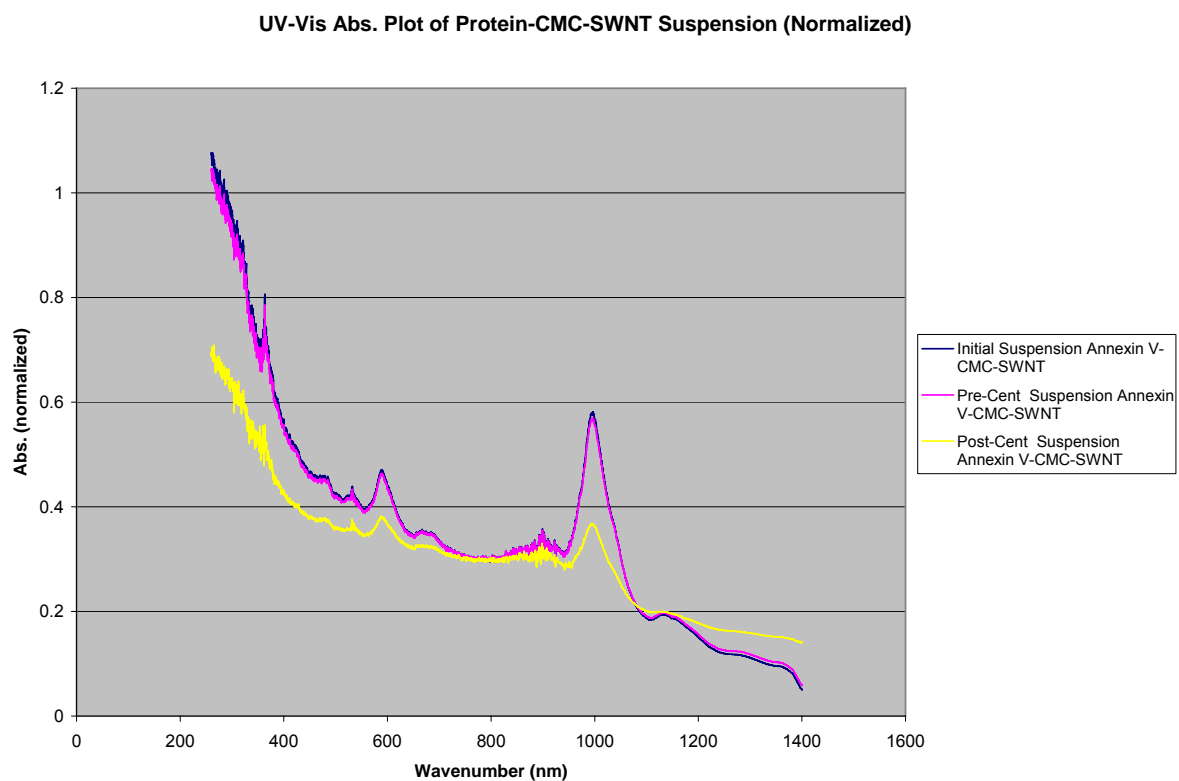
**Table 14:** Protein concentration results.

<b>Sample</b>	<b>Protein concentration, mg/L</b>	<b>SWNT Concentration, mg/L</b>	<b><u>Protein Weight</u>, mg/mg <u>SWNT Weight</u></b>
SWNT-CMC-annexin V suspension after centrifugation	74	14.6	5.1
Final dialysis solution (2 L) using 100 kDa membrane	0	–	–

**Table 15:** SWNT concentrations, as measured by the absorbance at 800 nm.

<b>Compound</b>	<b>SWNT concentration, mg/L</b>	<b>Yield, %</b>
Pristine SWNTs	26.6	100.0
SWNT-protein, pre-centrifugation	25.6	200.5
SWNT-protein, post-centrifugation*	14.6	113.4

\*From Table 14 data, protein weight/ SWNT weight = 5100  $\mu\text{g}/\text{mg}$  (= 141.67 nmol protein/mg SWNTs)



**Figure 68:** UV-Vis absorbance spectrum. An UV-Vis spectrophotometer (UV-2100 PC Shimadzu) was used to investigate the optical transitions in the wavelength range 300-1400 nm.

## **Future studies**

As discussed above, there are data available in the literature that supports the idea that annexin V plays an important role in the targeting of cancer cells. That principle can be used in order to try to develop a new therapeutic system. A non-term goal is to attach annexin V to SWNTs and retain the ability of annexin V to bind to PS.

*In vitro* studies using human endothelial cells with PS induced to be exposed on the cell surface and *in vivo* tests in mice with tumor xenografts are some of the proposed studies that try to explain and to understand the viability of this system when incorporated *in vivo*.



## **Conclusion**

The SWNT can be exploited as molecular transporters for various cargos. The biocompatibility, unique physical, electrical, optical, and mechanical properties of SWNTs provide the basis for new classes of materials for drug, protein, and gene delivery applications.

Evaluating the results, the adsorption of HRP on SWNTs was successful using the two different attachment methods. When HRP adsorption was performed using EDC as a crosslinker agent, there was some loss of activity compared to the native enzyme (enzyme activity/enzyme activity of native protein = 0.81). However the protein weight/SWNT weight was 36% higher than when HRP was attached by adsorption.

Although it was not possible to adsorb annexin V to SWNTs and keep the SWNTs suspended, the attachment of annexin V to SWNT-CMC using EDC and NHS was successful. Using this covalent attachment method, there was with an increase of 70% of protein loading compared to the same experiment using HRP.

The results obtained in the present study, using annexin V and HRP provide good motivation for conduct the studies proposed in this dissertation.

## LIST OF REFERENCES

Adler, R. R., A. K. Ng, et al. (1995). "Monoclonal Antiphosphatidylserine Antibody Inhibits Intercellular Fusion of the Choriocarcinoma Line, Jar." Biology of Reproduction **53**(4): 905-910.

Alcamo, I. E. (2001). DNA Technology - The awesome skill. New York, Academic Press.

Asenjo, J. A., Andrews, B. A. (1996). Recombinant DNA biotechnology III: the integration of biological and engineering sciences. New York, New York Academy of Sciences.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (1987). Current Protocols in Molecular Biology. New York, John Wiley & Sons.

Bachilo, S. M., L. Balzano, et al. (2003). "Narrow (n,m)-distribution of single-walled carbon nanotubes grown using a solid supported catalyst." Journal of the American Chemical Society **125**(37): 11186-11187.

Bailon, P., Ehrlich, G. K., Fung, W., and Berthold W. (2000). Affinity Chromatography, Humana Press.

Bandorowicz-Pikula, J. (2003). Annexins-biological importance and annexin-related pathologies. Texas, Kluwer Academic.

Barton, G. J., R. H. Newman, et al. (1991). "Amino-Acid-Sequence Analysis of the Annexin Supergene Family of Proteins." European Journal of Biochemistry **198**(3): 749-760.

Beck, A. W., T. A. Luster, et al. (2006). "Combination of a monoclonal anti-phosphatidylserine antibody with gemcitabine strongly inhibits the growth and metastasis of orthotopic pancreatic tumors in mice." International Journal of Cancer **118**(10): 2639-2643.

Benz, J. and A. Hofmann (1997). "Annexins: From structure to function." Biological Chemistry **378**(3-4): 177-183.

Bevers, E. M., P. Comfurius, et al. (1983). "Changes in Membrane Phospholipid Distribution during Platelet Activation." Biochimica Et Biophysica Acta **736**(1): 57-66.

Bianco, A., K. Kostarelos, et al. (2005). "Biomedical applications of functionalized carbon nanotubes." Chemical Communications(5): 571-577.

Biosciences, A. (2002). Affinity Chromatography - Principles and Methods.

Biosciences, B. (2007). "BD Biosciences Product Catalog."

Blankenberg, F. G., P. D. Katsikis, et al. (1998). "In vivo detection and imaging of phosphatidylserine expression during programmed cell death." Proceedings of the National Academy of Sciences of the United States of America **95**(11): 6349-6354.

Blondelle, S. E. (2006). Understanding Biology Using Peptides: Proceedings of the 19th American Peptide Symposium. New York, Springer.

Bloom, M. V., Freyer, G. A., Micklos, D. A. (1995). Laboratory DNA Science: an introduction to recombinant DNA techniques and methods of genome analysis. New York, The Benjamin/Cummings Publishing Company, Inc.

Bombeli, T., A. Karsan, et al. (1997). "Apoptotic vascular endothelial cells become procoagulant." Blood **89**(7): 2429-2442.

Bradford, M. M. (1976). "Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding." Analytical Biochemistry **72**(1-2): 248-254.

Brush, M. D. (2000). "Recourse to Death - A bevy of new products harnesses the power of flow cytometry for detecting apoptosis." The Scientist **14**(16): 25.

Buffa, F., H. Hu, et al. (2005). "Side-wall functionalization of single-walled carbon nanotubes with 4-hydroxymethylaniline followed by polymerization of epsilon-caprolactone." Macromolecules **38**(20): 8258-8263.

Burger, A., R. Berendes, et al. (1993). "A Rapid and Efficient Purification Method for Recombinant Annexin-V for Biophysical Studies." Febs Letters **329**(1-2): 25-28.

Catalog, W. P. (2006).

Celio, M. R., Pauls, T. L., Schwaller, B. (1996). Guidebook to the Calcium-Binding Proteins. Switzerland, Oxford University Press.

Cherukuri, P., C. J. Gannon, et al. (2006). "Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence." Proceedings of the National Academy of Sciences of the United States of America **103**(50): 18882-18886.

Cooper, G. M., Hausman, R. E. (2004). The Cell - A Molecular Approach. Washington, D. C., ASM Press.

- Crumpton, M. J. (1992). The Annexins. Cambridge, Portland Press.
- Daleke, D. L. and W. H. Huestis (1985). "Incorporation and Translocation of Aminophospholipids in Human-Erythrocytes." Biochemistry **24**(20): 5406-5416.
- Davis, G. D., Elisee, C., Newham, D. M., Harrison, R. G. (1999). "New fusion protein systems designed to give soluble expression in Escherichia coli." Biotechnology and Bioengineering **65**: 382-388.
- Dechow, F. J. (1989). Separation and Purification Techniques in Biotechnology. New Jersey, Noyes Publications.
- DeFrancesco, L. (1997). "The death of a cell: a profile of apoptosis kits and reagents." The Scientist **11**(24): 22.
- DeFrancesco, L. (1999). "Dead Again: Adventures in Apoptosis." **13**(5): 17.
- Demo, S. D., E. Masuda, et al. (1999). "Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay." Cytometry **36**(4): 340-348.
- Diaz, C. and A. J. Schroit (1996). "Role of translocases in the generation of phosphatidylserine asymmetry." Journal of Membrane Biology **151**(1): 1-9.
- Dieffenbach, C. W., Dveksler, G. S. (2003). PCR Primer: A laboratory manual. New York, Cold Spring Harbor Laboratory Press.
- Fernandez, J. M. (1999). Gene Expression Systems. California, Academic Press.
- Fink, D. J. (1979). "Cancer Overview." Cancer Research **39**(7): 2819-2821.
- Flaherty, M. J., West, S., Heimark, R. L., Fujiwaka, K., Tait, J. F. (1990). "Placental anticoagulant protein-I: measurement in extracellular fluids and cells of the hemostatic system." Journal of Laboratory and Clinical Medicine **115**: 174-181.
- Fu, K., Huang, W., Lin, Y., Zhang, D., Hanks, T. W., Rao, A. M., and Sun, Y. (2002). "Functionalization of Carbon Nanotubes with Bovine Serum Albumin in Homogeneous Aqueous Solution." Journal of Nanoscience and Nanotechnology **2**(5): 457-461.
- GE-Healthcare (2006). Recombinant Protein Purification Handbook.
- Geisow, M. J. (1986). "Common domain structure of Ca<sup>2+</sup> and lipid-binding proteins." Febs Letters **203**(1): 99-103.

Glick, B. R., Pasternak, J. J. (1998). Molecular Biotechnology: Principles and Applications of Recombinant DNA. Washington DC, ASM Press.

Gordesky, S. E., G. V. Marinetti, et al. (1975). "Reaction of Chemical Probes with Erythrocyte-Membrane." Journal of Membrane Biology **20**(1-2): 111-132.

Grabarek, Z. a. G., J. (1990). "Zero-length crosslinking procedure with the use of active esters." Analytical Biochemistry **185**: 131-5.

Graff, R. A., J. P. Swanson, et al. (2005). "Achieving individual-nanotube dispersion at high loading in single-walled carbon nanotube composites." Advanced Materials **17**(8): 980-+.

Griffiths, A. J. F., Gelbart, W. M., Miller, J. H., Lewontin, R. C. (2000). Modern Genetic Analysis. New York, W. H. Freeman and Company.

Harrison, R. G., P. Todd, et al. (2003). Bioseparations Science and Engineering. New York, Oxford University Press.

Heizmann, C. W. (1991). Novel calcium-binding proteins - Fundamentals and Clinical Implications. Berlin, Springer-Verlag.

Heizmann, C. W., Hunziker, W. (1990). Intracellular calcium binding molecules. New York.

Herrmann, A. and P. F. Devaux (1990). "Alteration of the Aminophospholipid Translocase Activity during In vivo and Artificial Aging of Human Erythrocytes." Biochimica Et Biophysica Acta **1027**(1): 41-46.

<http://data.engin.umich.edu/umseds/kc135/nanotubes/report/FinalReport.htm>.

<http://publications.csail.mit.edu/abstracts/abstracts06/kathryn/hrp.jpg>.

[http://science.nasa.gov/headlines/y2002/15jan\\_nano.htm](http://science.nasa.gov/headlines/y2002/15jan_nano.htm).

[http://us.expasy.org/tools/pi\\_tool.html](http://us.expasy.org/tools/pi_tool.html).

<http://www.azonano.com/details.asp?ArticleID=980> "Carbon Nanotube Applications (Internet Reference)."

<http://www.cheaptubesinc.com/applications.htm>. "Properties and Applications of Carbon Nanotubes."

<http://www.dinalfa.com>. "pET-30 Ek/LIC vector kit."

<http://www.emdbiosciences.com/docs/NDIS/69024-000.html>.

<http://www.lsbu.ac.uk/water/hycmc.html>.

<http://www.medicinenet.com/>.

<http://www.molecula.com/IPTG.gif>.

<http://www.molecularimaging.vcu.edu/>.

[http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=468888#sequence\\_468888](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=468888#sequence_468888).

[http://www.springboard4health.com/books\\_online/ps/phosphatidylserine.html](http://www.springboard4health.com/books_online/ps/phosphatidylserine.html).

[http://www.uni-leipzig.de/~strotm/molbio/vectors/vector\\_information.php?name=pET-30%20Ek-LIC](http://www.uni-leipzig.de/~strotm/molbio/vectors/vector_information.php?name=pET-30%20Ek-LIC).

<http://www.wikipedia.org/>.

Huang, W., Taylor, S., Fu, K., Lin, Y., Zhang, D., Hanks, T. W., Rao, A. M., and Sun, Y. (2002). "Attaching Proteins to Carbon Nanotubes via Diimide-Activated Amidation." Nanoletters **2**(4): 311-314.

Huang, X. M., M. Bennett, et al. (2005). "A monoclonal antibody that binds anionic phospholipids on tumor blood vessels enhances the antitumor effect of docetaxel on human breast tumors in mice." Cancer Research **65**(10): 4408-4416.

Huber, R., Berendes, R., Burger, A., Luecke H, Karshikov, A. (1992). "Annexin V-crystal structure and its implications on function." Behring Inst Mitt **91**: 107-25.

Huber, R., J. Romisch, et al. (1990). "The Crystal and Molecular-Structure of Human Annexin-V, an Anticoagulant Protein That Binds to Calcium and Membranes." Embo Journal **9**(12): 3867-3874.

Huber, R., M. Schneider, et al. (1990). "The Calcium-Binding Sites in Human Annexin-V by Crystal-Structure Analysis at 2.0 a Resolution - Implications for Membrane-Binding and Calcium-Channel Activity." Febs Letters **275**(1-2): 15-21.

Kam, N. W. S., M. O'Connell, et al. (2005). "Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction." Proceedings of the National Academy of Sciences of the United States of America **102**(33): 11600-11605.

- Karajanagi, S. S., Vertegel, A. A., Kane, R. S., and Dordick, J. S. (2004). "Structure and Function of Enzymes Adsorbed onto Single-Walled Carbon Nanotubes." Langmuir **20**: 11594-11599.
- Klee, C. B. (1988). "Ca<sup>2+</sup>-Dependent Phospholipid-Binding (and Membrane-Binding) Proteins." Biochemistry **27**(18): 6645-6653.
- Kling, J. (1997). "Flow Cytometry: It's Not Just For Immunologists Anymore." The Scientist **11**(13): 14.
- Kohli, P. and C. R. Martin (2005). "Smart nanotubes for biotechnology." Current Pharmaceutical Biotechnology **6**(1): 35-47.
- Kreuzer, H., Massey, A. (2001). Recombinant DNA and Biotechnology. Washington, DC, ASM Press.
- Liemann, S. and R. Huber (1997). "Three-dimensional structure of annexins." Cellular and Molecular Life Sciences **53**(6): 516-521.
- Lin, Y., S. Taylor, et al. (2004). "Advances toward bioapplications of carbon nanotubes." Journal of Materials Chemistry **14**(4): 527-541.
- Liu, Y. and H. Wang (2007). "Nanotechnology tackles tumors." Nature Nanotechnology **2**.
- Liu, Z., W. B. Cai, et al. (2007). "In vivo biodistribution and highly efficient tumor targeting of carbon nanotubes in mice." Nature Nanotechnology **2**(1): 47-52.
- Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., Darnell, J. (1999). Molecular Cell Biology. New York, W. H. Freeman and Company.
- Lolli, G., L. A. Zhang, et al. (2006). "Tailoring (n,m) structure of single-walled carbon nanotubes by modifying reaction conditions and the nature of the support of CoMo catalysts." Journal of Physical Chemistry B **110**(5): 2108-2115.
- Lupu, F., N. Moldovan, et al. (1993). "Intrinsic Procoagulant Surface-Induced by Hypercholesterolemia on Rabbit Aortic Endothelium." Blood Coagulation & Fibrinolysis **4**(5): 743-752.
- Marinetti, G. V. and R. C. Crain (1978). "Topology of Amino-Phospholipids in Red-Cell Membrane." Journal of Supramolecular Structure **8**(2): 191-213.
- Matsuura K, S. T., Okazaki T, Ohshima S, Yumura M and Iijima S (2006). "Selectivity of water-soluble proteins in single-walled carbon nanotube dispersions." Chemical Physics Letters **429**: 497-502.

- McDonald, D. M. and P. Baluk (2002). "Significance of blood vessel leakiness in cancer." Cancer Research **62**(18): 5381-5385.
- Mira, J. P., T. Dubois, et al. (1997). "Inhibition of cytosolic phospholipase A(2) by annexin V in differentiated permeabilized HL-60 cells - Evidence of crucial importance of domain I type II Ca<sup>2+</sup>-binding site in the mechanism of inhibition." Journal of Biological Chemistry **272**(16): 10474-10482.
- Novagen, I. (1994). "inNovations – Newsletter of Novagen, Inc." **1**(1).
- Novagen, I. (2004). "HRV 3C Protease - User Protocol TB420." 7.
- Novagen, I. (2006). "Novagen Catalog."
- Novagen, I. (2006). "pET System Manual." **11**.
- Ohsawa, K., Y. Imai, et al. (1996). "Molecular cloning and characterization of annexin V-binding proteins with highly hydrophilic peptide structure." Journal of Neurochemistry **67**(1): 89-97.
- Orr, F. W., H. H. Wang, et al. (2000). "Interactions between cancer cells and the endothelium in metastasis." Journal of Pathology **190**(3): 310-329.
- Palwai, N. R., Martyn, D. E., Neves, L. F. F., Tan, Y., Resasco, D. E., and Harrison, R. G. (2007). "Adsorption of Horseradish Peroxidase on Single-Walled Carbon Nanotubes: Retention of Biological Activity and Near-Infrared Absorbance." Nanotechnology **18**: 235601.
- Qu, J. M., L. A. Conroy, et al. (1996). "Phosphatidylserine-mediated adhesion of T-cells to endothelial cells." Biochemical Journal **317**: 343-346.
- Ran, S., A. Downes, et al. (2002). "Increased exposure of anionic phospholipids on the surface of tumor blood vessels." Cancer Research **62**(21): 6132-6140.
- Ran, S. and P. E. Thorpe (2002). "Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy." International Journal of Radiation Oncology Biology Physics **54**(5): 1479-1484.
- Rao, L. V. M., J. F. Tait, et al. (1992). "Binding of Annexin-V to a Human Ovarian-Carcinoma Cell-Line (Oc-2008) - Contrasting Effects on Cell-Surface Factor-Viia/Tissue Factor Activity and Prothrombinase Activity." Thrombosis Research **67**(5): 517-531.
- Rauch, J. and A. S. Janoff (1990). "Phospholipid in the Hexagonal-Ii Phase Is Immunogenic - Evidence for Immunorecognition of Nonbilayer Lipid Phases Invivo."



Proceedings of the National Academy of Sciences of the United States of America **87**(11): 4112-4114.

Rodriguez, R. L., Denhardt, D. T. (1987). Vectors: a survey of molecular cloning vectors and their uses. Stoneham, MA, Butterworth Publishers.

Romisch, J. and E. P. Paques (1991). "Annexins - Calcium-Binding Proteins of Multifunctional Importance." Medical Microbiology and Immunology **180**(3): 109-126.

Rothe, G. M. (1994). Electrophoresis of Enzymes - Laboratory Methods. Berlin, Springer Verlag.

Russell, P. J. (1998). Genetics. New York, The Benjamin/Cummings Publishing Company, Inc.

Sambrook, J., Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanchez, J.-C., Corthals, G. L., Hochstrasser, D. F. (2004). Biomedical Applications of Proteomics. Switzerland, Wiley-VCH.

Schlaepfer, D. D. and H. T. Haigler (1990). "Expression of Annexins as a Function of Cellular Growth-State." Journal of Cell Biology **111**(1): 229-238.

Schönthal, A. H. (2004). Checkpoint Controls and Cancer, Humana Press.

Scopes, R. (1982). Protein Purification: Principles and practice. New York, Springer-Verlag.

Seaton, B. A., J. F. Head, et al. (1990). "Purification, Crystallization, and Preliminary-X-Ray Diffraction Analysis of Rat-Kidney Annexin-V, a Calcium-Dependent Phospholipid-Binding Protein." Journal of Biological Chemistry **265**(8): 4567-4569.

Seigneuret, M. and P. F. Devaux (1984). "Atp-Dependent Asymmetric Distribution of Spin-Labeled Phospholipids in the Erythrocyte-Membrane - Relation to Shape Changes." Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **81**(12): 3751-3755.

Sessions, A. and A. F. Horwitz (1981). "Myoblast Aminophospholipid Asymmetry Differs from That of Fibroblasts." Febs Letters **134**(1): 75-78.

Shaughnessy, S. G., M. R. Buchanan, et al. (1989). "Walker Carcinosarcoma Cells Damage Endothelial-Cells by the Generation of Reactive Oxygen Species." American Journal of Pathology **134**(4): 787-796.

- Sherbet, G. V. (2000). Calcium signaling in cancer, CRC.
- Sigel, A. and H. Sigel (2004). Metal Complexes in Tumor Diagnosis and as Anticancer Agents. Switzerland, Marcel Dekker, Inc.
- Smith, V. L., M. A. Kaetzel, et al. (1990). "Stimulus-Response Coupling - the Search for Intracellular Calcium Mediator Proteins." Cell Regulation **1**(2): 165-172.
- Soares, F. A., S. G. Shaughnessy, et al. (1994). "Quantification and Morphologic Demonstration of Reactive Oxygen Species Produced by Walker-256 Tumor-Cells in-Vitro and during Metastasis in-Vivo." Laboratory Investigation **71**(4): 480-489.
- Society, A. C. (2006). Cancer Facts & Figures.
- Staros, J. V., et al. (1986). "Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions." Analytical Biochemistry **156**: 220-2.
- Stratton, J. R., Dewhurst, T. A., Kasina, S., Reno, J. M., Cerqueira, M. D., Baskin, D. G., Tait, J. F. (1995). "Circulation selective uptake of radiolabeled annexin V on acute porcine left atrial thrombi." Circulation **92**: 3113-3121.
- Tait, J. F., M. D. Cerqueira, et al. (1994). "Evaluation on Annexin-V as a Platelet-Directed Thrombus Targeting Agent." Thrombosis Research **75**(5): 491-501.
- Tait, J. F., S. Engelhardt, et al. (1995). "Prourokinase-Annexin-V Chimeras - Construction, Expression, and Characterization of Recombinant Proteins." Journal of Biological Chemistry **270**(37): 21594-21599.
- Tait, J. F., C. Smith, et al. (2002). "Development of annexin V mutants suitable for labeling with Tc(I)-carbonyl complex." Bioconjugate Chemistry **13**(5): 1119-1123.
- Thiagarajan, P. and J. F. Tait (1990). "Binding of Annexin-V Placental Anticoagulant Protein I to Platelets - Evidence for Phosphatidylserine Exposure in the Procoagulant Response of Activated Platelets." Journal of Biological Chemistry **265**(29): 17420-17423.
- Thiagarajan, P. and J. F. Tait (1991). "Collagen-Induced Exposure of Anionic Phospholipid in Platelets and Platelet-Derived Microparticles." Journal of Biological Chemistry **266**(36): 24302-24307.
- Timkovich, R. (1977). "Detection of the stable addition of carbodiimide to proteins." Analytical Biochemistry **79**: 135-43.
- Utsugi, T., A. J. Schroit, et al. (1991). "Elevated Expression of Phosphatidylserine in the Outer-Membrane Leaflet of Human Tumor-Cells and Recognition by Activated Human Blood Monocytes." Cancer Research **51**(11): 3062-3066.

Vangeelen, A. G. M., M. E. P. Slobbevandrunen, et al. (1995). "Membrane Related Effects in Endothelial-Cells Induced by Human Cytomegalovirus." Archives of Virology **140**(9): 1601-1612.

Verhoven, B., R. A. Schlegel, et al. (1995). "Mechanisms of Phosphatidylserine Exposure, a Phagocyte Recognition Signal, on Apoptotic T-Lymphocytes." Journal of Experimental Medicine **182**(5): 1597-1601.

Verkleij, A. J., R. F. A. Zwaal, et al. (1973). "Asymmetric Distribution of Phospholipids in Human Red-Cell Membrane - Combined Study Using Phospholipases and Freeze-Etch Electron-Microscopy." Biochimica Et Biophysica Acta **323**(2): 178-193.

Vogt, E., A. K. Ng, et al. (1996). "A model for the antiphospholipid antibody syndrome: Monoclonal Antiphosphatidylserine antibody induces intrauterine growth restriction in mice." American Journal of Obstetrics and Gynecology **174**(2): 700-707.

Wang, F., X. W. He, et al. (2006). "Non-fusion expression in Escherichia coli: Single-step purification of recombinant human annexin A5 for detection of apoptosis." Protein Expression and Purification **45**(1): 80-87.

Welinder, K. G. (1979). "Amino-Acid Sequence Studies of Horseradish-Peroxidase .4. Amino and Carboxyl Termini, Cyanogen-Bromide and Tryptic Fragments, the Complete Sequence, and Some Structural Characteristics of Horseradish Peroxidase-C." European Journal of Biochemistry **96**(3): 483-502.

Yuan, H., X. Yang, et al. (2004). "Optimization of expression of an Annexin V-Hirudin chimeric protein in Escherichia coli." Microbiological Research **159**(2): 147-156.

Zulueta, J. J., F. S. Yu, et al. (1995). "Release of Hydrogen-Peroxide in Response to Hypoxia-Reoxygenation - Role of an Nad(P)H Oxidase-Like Enzyme in Endothelial-Cell Plasma-Membrane." American Journal of Respiratory Cell and Molecular Biology **12**(1): 41-49.

Zwaal, R. F. A., B. Roelofsen, et al. (1975). "Organization of Phospholipids in Human Red-Cell Membranes as Detected by Action of Various Purified Phospholipases." Biochimica Et Biophysica Acta **406**(1): 83-96.

## APPENDIX A

### Laboratory Protocols

#### A.1. Reagent Protocols

##### A.1.1. Luria Bertani (LB) media

**Table A.1:** LB media composition (Ausubel 1987).

Component	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
1N NaOH	1 ml
Add dH <sub>2</sub> O to 1.0 L and autoclave (25 mins).	

A.1.2. LB agar

**Table A.2:** LB Agar / SOC media composition (per Liter).

Component	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
Add dH <sub>2</sub> O to 1.0 L	
Adjust pH to 7.0 with 5 N NaOH	
Autoclave	
Pour into Petri dishes	
(~25 ml/100-mm plate)	

### A.1.3. Additives:

Antibiotic: Kanamycin ( $C_{18}H_{36}N_4O_{11} \cdot H_2SO_4$ )

Galactosides: IPTG to 0.1 mM

**Table A.3:** Kanamycin properties (Ausubel 1987).

Antibiotic <sup>a</sup>	Stock conc. (mg/ml)	Final conc. ( $\mu$ g/ml)	Mode of action	Mode of resistance
Kanamycin	10	30	Bacteriocidal; inhibits protein synthesis; inhibits translocation and elicits miscoding.	Aminoglycoside phosphotransferase also know as neomycin phosphotransferase, aminoglycoside acetyltransferase, and aminoglycoside nucleotidyltransferase; inactivates kanamycin.

<sup>a</sup> Kanamycin should be stored at 4 °C, since it loses potency at room temperature (Ausubel 1987).

#### A.1.4. Kanamycin preparation

To prepare kanamycin (10 mg/ml) 25 ml of deionized water and 250 mg of kanamycin (Sigma, St. Louis, MO) was mixed, and saved in the freezer (-20 °C).

A.1.5. IPTG preparation (10 mg/ml)

0.4 g of IPTG (stored at -20 °C)

40 ml of sterile and distilled water



#### A.1.6. Sonication buffer preparation

0.05 mM N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)

1 mM phenylmethylsulfonyl fluoride (PMSF)

1 % of ethanol (ethyl alcohol)

0.01 %  $\beta$ -mercaptoethanol

0.02 M of sodium phosphate

The pH was adjusted to 7.4.

#### A.1.7. Sonication protocol (4 °C)

The sonication equipment (Sonic Dismembrator 550 (Fisher Scientific, USA)) is turned on to the position 4/5. The tip of the sonicator (Sonic Dismembrator 550 (Fisher Scientific, USA)) is immersed in the solution to disrupt the cell membranes for 30 seconds. After that, the sonicator is turned off for another 30 seconds (with the beaker placed on ice). This procedure is repeated 4x corresponding to a total sonication time of 2.5 minutes. After the sonication, the solution is centrifuged at 12,000 x g for 30 min.



**Figure A.1:** Sonication process.

### A.1.8. HRV 3C protease

Recombinant type 14 3C protease from human rhinovirus (HRV 3C) is a recombinant restriction-grade protease. HRV 3C is a highly purified recombinant 6X His-fusion protein, that recognizes the same cleavage site as the native enzyme: Leu Glu Val Leu Phe Gln ↓ Gly Pro. The small, 22-kDa size of the protease, optimal activity at 4 °C, high specificity, and His-Tag<sup>®</sup> fusion make HRV 3C protease an ideal choice for rapid removal of fusion tags (Novagen 2006).

#### **Components**

- 500 U      HRV 3C Protease (2000 U/ml in 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM THP, 50% glycerol, pH 8.0)
- 10 µg      HRV 3C Cleavage Control Protein (in 100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, 50% glycerol, pH 7.4)
- 10 ml      10X HRV 3C Protease Cleavage Buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5)

Unit definition: One unit will cleave > 95 % of 100 µg His-Tag fusion control protein in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 at 4 °C for 16 hours.

### A.1.9. Tris-HCl

1.5 M Tris-HCl pH 8.8 (TRIS – Electrophoresis purity reagent (Tris(hydroxymethyl)-aminomethane) (CA, USA))

1 M Tris-HCl pH 6.8 (TRIS – Electrophoresis purity reagent (Tris(hydroxymethyl)-aminomethane) (CA, USA))

A.1.10. Acrylamide (29%) Bis (1%)

Mix 29.0 g acrylamide and 1.0 g N-N'-methylene-bisacrylamide in a total volume of 100 ml H<sub>2</sub>O. Store at 4 °C in the dark. Discard after 30 days, since acrylamide gradually hydrolyzes to acrylic acid and ammonia.

*CAUTION: Acrylamide monomer is neurotoxic. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.*

A.1.11. Electrophoresis buffer

10% Bio-Rad 10x Tris/Glycine/SDS Buffer (Bio-Rad Laboratories (Hercules, CA))

90% H<sub>2</sub>O

A.1.12. Ammonium persulfate preparation

10 g ammonium persulfate

H<sub>2</sub>O to 100 ml total volume

Store refrigerated ~ 2 weeks.

## **A.2 Experimental Protocols**

### **A.2.1. Transformation Protocol**

#### **Transformation**

NovaBlue GigaSingles™ Competent Cells (Cat. No. 71127) are provided in the Ek/LIC Vector Kits and should be used for initial cloning with all Ek/LIC Vectors. NovaBlue is a convenient host for initial cloning of target DNA into the Ek/LIC Vector due to its high transformation efficiency and the high yields and excellent plasmid DNA that results from *recA endA* mutations. Single-use NovaBlue GigaSingles Competent Cells are provided in 50-µl aliquots. The pET, pCDF-2, pRSF-2, and pTriEx™ Ek/LIC Vector Kits also receive expression host strains in standard, 0.2 ml aliquots.

The standard transformation reaction calls for 20 µl of cells, so each tube contains enough cells for 10 transformations. The following protocol is appropriate for transformations using either GigaSingles or Standard Competent Cells with noted differences for the steps effected.

*Note: Upon receipt of competent cells from Novagen, verify that the cells are frozen and that dry ice is present in the shipping container. Immediately place the competent cells at -70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible. To mix cells, flick the tube 1–3 times. NEVER vortex competent cells.*

1. Remove the appropriate number of competent cell tubes from the freezer (include on extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells and gently flick the cells 1–2 times to evenly resuspend the cells.



### 3. *GigaSingles Kits:*

If a Test Plasmid sample is included, proceed to Step 4, if not go directly to Step 5.

#### *Standard Kits:*

Place the required number of 1.5-ml snapcap polypropylene tubes on ice to pre-chill.

Pipet 20  $\mu$ l aliquots of cells into the prechilled tubes.

4. (Optional) To determine transformation efficiency, add 1  $\mu$ l (0.2 ng) Test Plasmid to one of the tubes containing cells. Gently flick the tube to mix and return to the ice.

5. Add 1  $\mu$ l of the annealing reaction directly to the cells. Stir gently to mix and return to the ice.

Repeat for additional samples.

6. Incubate on ice for 5 min.

7. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

8. Place the tubes on ice for 2 min.

### 9. *GigaSingles Kits:*

Add **250  $\mu$ l** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

#### *Standard Kits:*

Add **80  $\mu$ l** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

10. Incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.

11. Selection for transformants is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance (50  $\mu$ g/ml carbenicillin or ampicillin for the Amp resistance marker, 30  $\mu$ g/ml kanamycin for the kan resistance marker, and 50

µg/ml of streptomycin and/or spectinomycin for the *aadA* gene). Additional host-specific antibiotics may also be appropriate to ensure maintenance of the host-encoded feature(s). When plating less than 25 µl, first pipet a “pool” of SOC onto the plate and then pipet the cells into the SOC. The appropriate amount of transformation mixture to plate varies with the efficiencies of the annealing process and of the competent cells (see certificate of analysis for efficiency). For recombinants in NovaBlue, expect 105–107 transformants/µg plasmid, depending on the particular insert and the ligation efficiency. For the Test Plasmid, plate only 5 µl of the NovaBlue transformation mix or 10 µl of any strain with a  $2 \times 10^6$  efficiency in a “pool” of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene, *bla*).

12. Set plates on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

### **Transformation Protocol for Experienced Users**

*Note: See the next section for a detailed protocol.*

1. Thaw the required number of tubes of cells on ice and mix gently to ensure that the cells are evenly suspended.

2. *Standard Kits:*

Place the required number of 1.5-ml polypropylene microcentrifuge tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes.

*Singles™ Kits:*

Proceed to Step 3.

3. Add 1 µl of the DNA solution directly to the cells. Stir gently to mix.

4. Place the tubes on ice for 5 min.

5. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

6. Place on ice for 2 min.

7. *Standard Kits:*

Add **80 µl** of room temperature SOC

Medium to each tube.

*Singles Kits:*

Add **250 µl** of room temperature SOC

Medium to each tube.

8. Selection for transformants is accomplished by plating on media containing antibiotic for the plasmid-encoded drug resistance. Additional host-specific antibiotics may also be appropriate to insure maintenance of the host-encoded feature(s).

**When using NovaBlue strain:** if selecting for ampicillin or chloramphenicol resistance, plate 5–50 µl cells directly on selective medium (plus IPTG/X-gal for plasmids that permit blue/white screening). If selecting for kanamycin or streptomycin/spectinomycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating on selective medium.

**When using strains other than NovaBlue:** incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.

### A.2.2. Bradford protein assay

#### Principle

This assay is a dye-binding assay in which a differential color change of dye occurs in response to various concentrations of protein. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine.

#### Protocol

##### Mix:

- 20  $\mu$ l of enzyme sample
- 780  $\mu$ l of water
- 200  $\mu$ l of concentrated Bio-Rad reagent

Incubate at room temperature for 10 minutes. Measure the absorbance at 595 nm.

Spectrophotometry is the experimental technique that analyzes and quantifies the absorbance (light absorption). This technique requires a spectrophotometer, which can quantify intensity as a function of the light wavelength.

The spectrophotometers can be classified as single beam or double beam. In this experimental work, a double beam spectrophotometer was used, that measures the absolute light intensity. Usually they are equipped with a monochromator that analyses the spectrum.

Basically, the spectrophotometer quantifies the fraction of light that passes through a given solution. The intensity of the remaining light is determined using a photodiode, and the transmittance of the wavelength is calculated automatically.

#### Standard

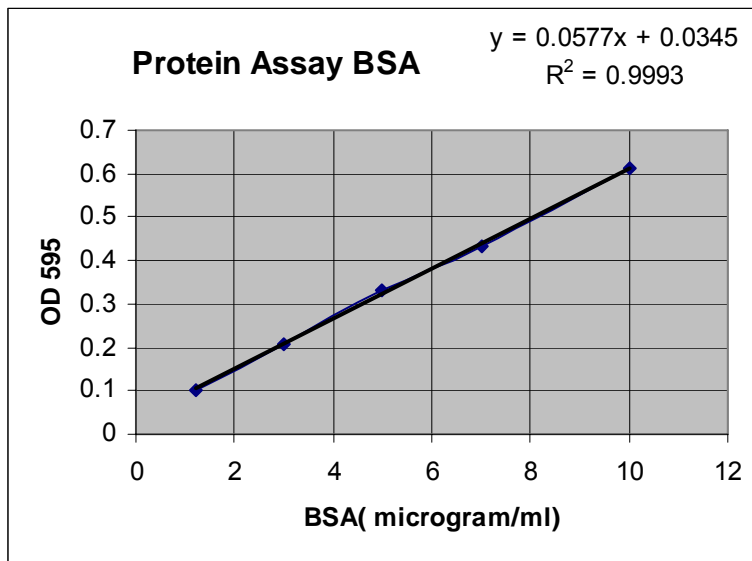
Use the standard below to calculate the protein concentration.

$$y = 0.0577x + 0.0345$$

$$R^2 = 0.993$$

y – OD 595

x – Protein Concentration ( $\mu\text{g/ml}$ )

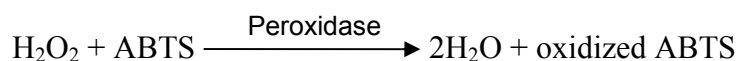


**Figure A.2:** Bradford protein assay.

### A.2.3. Enzymatic assay of peroxidase

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate

#### **PRINCIPLE:**



Abbreviation used:

ABTS<sup>1</sup> = 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)

**CONDITIONS:** T = 25 °C, pH = 5.0, A<sub>405nm</sub>, Light path = 1 cm

**METHOD:** Continuous Spectrophotometric Rate Determination

#### **REAGENTS:**

A. 100 mM potassium phosphate buffer, pH 5.0 at 25 °C

(Prepare 100 ml in deionized water using potassium phosphate, monobasic, Sigma Prod. No. P-5379. Adjust to pH 5.0 at 25 °C using 1.0 M KOH.)

B. 9.1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) Substrate Solution (ABTS<sup>1</sup>)

(Prepare 30 ml in Reagent A using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Tablets Diammonium Salt, Sigma Prod. No. A-9941. **PREPARE FRESH.**)

C. 0.3% (w/w) hydrogen peroxide solution (H<sub>2</sub>O<sub>2</sub>)

(Prepare 50 ml in deionized water using hydrogen peroxide, 30% (w/w) solution, Sigma Prod. No. H-1009. **PREPARE FRESH.**)

D. 40 mM potassium phosphate buffer with 0.25% (w/v) bovine serum albumin and 0.5% (v/v) Triton X-1002, pH 6.8 at 25 °C (Enzyme Diluent)

(Prepare 100 ml in deionized water using potassium phosphate, monobasic, Sigma Prod. No. P-5379, albumin, bovine, Sigma Prod. No. A-4503, Triton X-100, Sigma Stock No. X-100. Adjust to pH 6.8 at 25 °C using 1 M KOH.)

E. Peroxidase Enzyme Solution

(Prepare an enzyme stock solution containing 10 mg/ml in cold Reagent D. Immediately before use, prepare a solution containing 0.20 - 0.80 unit/ml of Peroxidase in cold Reagent D.)

### PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reagent B (ABTS)	2.90	2.90
Reagent D (Enzyme Diluent)	-----	0.05
Reagent E (Enzyme Solution)	0.05	-----
Mix by inversion and equilibrate to 25 °C. Monitor the $A_{405\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:		
Reagent C (H <sub>2</sub> O <sub>2</sub> )	0.10	0.10

Immediately mix by inversion and record the increase in  $A_{405\text{nm}}$  for approximately 2 minutes. Obtain the  $\Delta A_{405\text{ nm}}/\text{minute}$  using the maximum linear rate for both the Test and Blank.

### **CALCULATIONS:**

$$\text{Units/ml enzyme} = \frac{(\Delta A_{405 \text{ nm}}/\text{min Test} - \Delta A_{405 \text{ nm}}/\text{min Blank})(3.05)(\text{df})}{(36.8)(0.05)}$$

3.05 = Total volume (in milliliters) of assay

df = Dilution factor

36.8 = Millimolar extinction coefficient of oxidized ABTS at 405 nm

0.05 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

### **UNIT DEFINITION:**

One unit will oxidize 1.0 mmole of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) per minute at pH 5.0 at 25 °C.

### **FINAL ASSAY CONCENTRATIONS:**

In a 3.05 ml reaction mix, the final concentrations are 96 mM potassium phosphate, 8.7 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.01% (w/w) hydrogen peroxide, 0.004% (w/v) bovine serum albumin, 0.008% (v/v) Triton X-100 and 0.01 - 0.04 unit peroxidase.

### **REFERENCE:**



Keesey, J. (1987) in Biochemica Information, pp. 58, First Edition, Boehringer Mannheim Biochemicals, Indianapolis, IN

Pütter, J. and Becker, R. (1983) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 3rd ed., Vol III, pp. 286-293, Verlag Chemie, Deerfield Beach, FL

**NOTES:**

1. ABTS is a registered trademark of Boehringer Mannheim GmbH.
2. Triton is a registered trademark of the Rohm & Haas Co.
3. The maximum linear rate occurs within the first minute of the reaction.
4. The millimolar extinction coefficient is cited in Keesey, J. (1987).
5. This assay is based on the cited references.
6. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

#### A.2.4. SDS-PAGE analysis of proteins

**Table A.4:** Reagents required for the casting of mini-SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels.

Components	Stacking gel 4%	Separating gel	
		12%	15%
dH <sub>2</sub> O	3.63 ml	3.34 ml	2.34 ml
1.5 M Tris-HCL pH 8.8	-	2.5 ml	2.5 ml
1 M Tris-HCL pH 6.8	625 µl	-	-
10 % (w/v) SDS	50 µl	100 µl	100 µl
Acrylamide (29%) Bis (1%)	667 µl	4 ml	5 ml
Ammonium persulfate 10%	25 µl	50 µl	50 µl
TEMED	5 µl	10 µl	10 µl
Total	5 ml	10 ml	10 ml

- Assemble the glass plates on the gel casting stand and fill with water to ensure that they are sealed.
- Mix the components of separating gel, adding the TEMED (N, N, N',N' – Tetramethylethylene-diamine, for electrophoresis, approx. 99% C<sub>6</sub>H<sub>16</sub>N<sub>2</sub>) last. Mix well and immediately fill the glass plates, leaving a 1.5 cm gap at the top (for the stacking gel).
- Immediately add 1 ml of isopropanol on top of the gel to prevent oxygen from inhibiting the polymerization. Wait 20 minutes for solidification.
- Pour off the isopropanol and rinse with dH<sub>2</sub>O to remove any residual isopropanol. Mix the components for the 4% stacking gel and pour on top of the separating gel. Insert the well-comb and wait 20 minutes for solidification.
- Preheat a water bath to 100 °C.

- Prepare the SDS-PAGE loading buffer by diluting  $\beta$ -mercaptoethanol 20 X (1:20) with the SDS-blue buffer.
- For a 4 ml culture with an approximate  $OD_{600}$  of 0.8, add 600  $\mu$ l of SLB to the cold, centrifuged pellet. Suspend pellet in the SLB by vortexing.
- Immediately heat the sample to 100°C for 2 minutes.
- Allow the samples to cool down for at least one minute before loading the gel.
- Assemble the solidified gels in the buffer chamber. Fill the chamber with running buffer.
- Load 10  $\mu$ l of each protein sample into the wells. Run the gel at a constant voltage of 165 V for about 1 hour, or until the dye reaches the bottom of the gel.
- Cut off the stacking gel and discard it.
- Stain the separating gel with a staining solution containing 45 % (w/v)  $dH_2O$ , 45% (w/v) methanol, 10 % (v/v) acetic acid and 0.25 % (w/v) Coomassie Brilliant Blue R250. Stain for 1 hour with gentle shaking. If shaking is not possible, an overnight incubation will be sufficient.
- Pour the stain back into its container (it can be re-used several times) and rinse the tray with water. To remove the extra stain on the gel, use a destain solution (same as the staining solution, but without the Coomassie Blue). Protein bands will be visible immediately on a light table.

**Table A.5:** Marker molecular weights (BIO-RAD Laboratories (Hercules, CA)).

Protein	Calibrated MW (daltons)	Calibrated MW (daltons)	Calibrated MW (daltons)
Myosin	202,816	196,496	196,373
$\beta$ -galactosidase	115,556	109,828	112,519
Bovine serum albumin	98,223	59,375	70,546
Ovalbumin	51,366	41,485	42,798
Carbonic anhydrase	37,240	28,224	31,635
Soybean trypsin inhibitor	29,003	20,261	
Lypozyme	19,748	15,013	
Aprotinin	6,658	6,494	

#### A.2.5. Freeze dryer protocol

1. Make sure that there is ethanol in the freezing well vacuum trap.
2. Turn on the vacuum trap and wait till red lights turns to green, indicating that ethanol in freezing well reached the appropriate temperature ( $\sim -110$  °C) (approx 1 h).
3. Turn on the vacuum pump. (Make sure there's oil in the pump)
4. Turn on the freeze dryer wait till the pressure drops (approx. 1 h).
  - a. Top light in the gauge reads higher pressures
  - b. Bottom light reads low pressures
  - c. Drain valve is completely closed
  - d. Vent valve is completely closed
5. Make sure that there is no leak in the freeze dryer. Presently only one vent is used to dry the samples, stoppers close all other vents.
6. Make sure the there is ice in the small Styrofoam box and place this box in the big Styrofoam box.
7. Place the filter paper under the freeze dryer cap (freeze dryer cap is connected to freeze dryer by a long tube).
8. Place the sample in the 20 ml freeze dryer vials (either in centrifuge tubes or other long tubes).
9. Place the 20 ml freeze dryer vial in the small Styrofoam box containing ice (make sure vial is completely immersed in ice).
10. Close the vial with black cap containing filter paper.
11. Open the vent valve on the freeze dryer.
12. Wait until the sample is completely freeze dried.
13. After complete drying, turn off all the equipment.
14. Open the drain valve on the freeze dryer, slowly to release the pressure.
15. Take the sample vial and store.

Note. Some times if the sample volume is large, sample will melt after few hours, if this is the case, use dry ice instead of ice in the Styrofoam box (This is due to inefficiency of the whole system).

## FREEZE DRYING

### FREEZE DRYING WITH RUBBER VALVES

1. Start the vacuum pump.  
The start switch is placed on top of the pump.
2. Open the gas ballast valve on the pump.
3. Check the oil level in the vacuum pump.  
The oil level glass must be  $\frac{1}{2}$  to  $\frac{1}{1}$  full.
4. Check the oil level in the oil mist filter.  
Drain the oil if the oil level glass is more than  $\frac{1}{2}$  full.
5. Check that the ice condenser is free from ice and water.
6. Check that the rubber valves are all closed, and that the grey cone is inserted in the top of the unit.
7. Check that the drain valve is closed.
8. Start the freeze dryer.  
WAIT.  
The cooling unit and vacuum pump should be allowed to run for 30 minutes before the freeze drying is started.
9. Open the vacuum valve.  
Wait for the pressure to drop below 0.5 mbar and for the temperature to drop below  $-30\text{ }^{\circ}\text{C}$ .
10. Connect the flask with the frozen material to the rubber valve, and slowly open the valve, turning your face away from the flask.  
The freeze drying (sublimation) is now running.  
Before connecting the next flask, wait for the pressure to drop below 0.5 mbar.

## **STOPPING THE FREEZE DRYING**

11. Turn the rubber valve handle 180° to pressure equalize the pressure in the flask, and remove the flask.
12. Close the vacuum valve after removal of the last flask.
13. Switch off the freeze dryer.

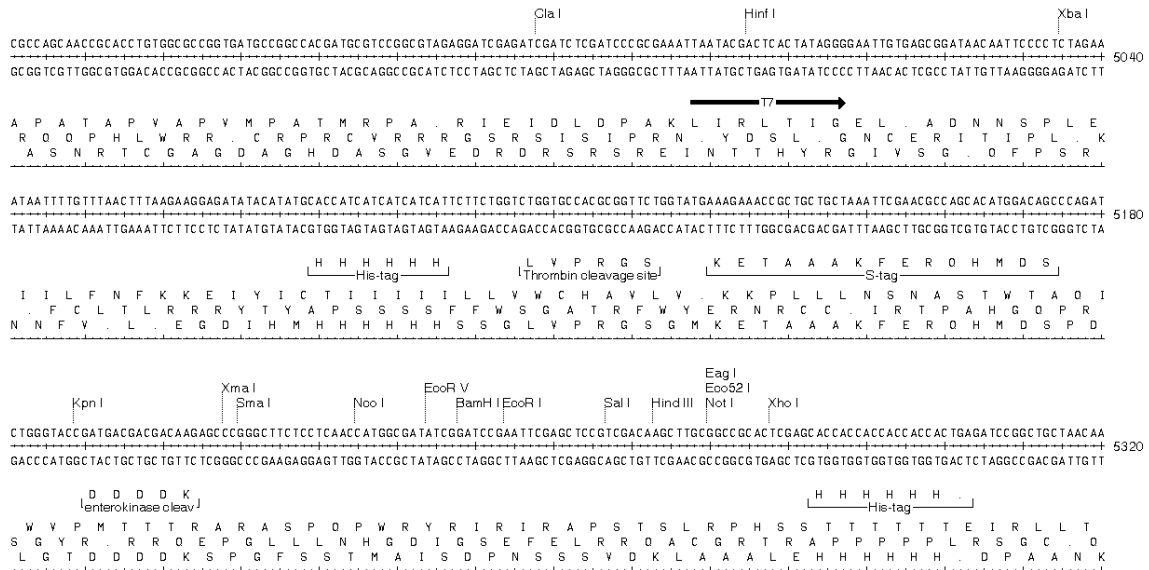
## APPENDIX B

### pET-30 Ek-LIC

<b>Info</b>	Prokaryotic expression of LIC-cloned PCR amplificates
<b>Selection</b>	Kanamycin
<b>Seq primers</b>	T7 / T7 term
<b>Source</b>	Novagen
<b>Sequence</b>	pET-30 Ek-LIC
<b>Notes</b>	LIC-cloning of PCR fragments for prokaryotic expression. The N-terminal His <sub>6</sub> - and S-tags can be cleaved off by thrombin and enterokinase, respectively. A C-terminal His <sub>6</sub> tag can be included with omission of the stop codon in the insert.



## Multiple cloning site



**Figure B.1:** pET-30 Ek/LIC vector map showing the multiple cloning site

([http://www.uni-leipzig.de/~strotm/molbio/vectors/vector\\_information.php?name=pET-30%20Ek-LIC](http://www.uni-leipzig.de/~strotm/molbio/vectors/vector_information.php?name=pET-30%20Ek-LIC)).

## APPENDIX C

LOCUS	pET30\Ek/L	5439 bp	DNA	circular	21-MAY-2002
SOURCE					
ORGANISM					
COMMENT	http://www.informaxinc.com				
COMMENT	This file is created by Vector NTI http://www.informaxinc.com/				
COMMENT	ORIGDB GenBank				
COMMENT	VNTDBDATE 267294239				
COMMENT	VNTNAME pET30 Ek/LIC				
COMMENT	VNTAUTHORNAME Demo User				
COMMENT	VNTAUTHORNAME Larissa Wiskowski				
COMMENT	VNTAUTHORTEL 608-238-6110				
COMMENT	VNTAUTHORFAX 608-238-1388				
COMMENT	VNTAUTHOREML Novatech@novagen.com				
COMMENT	VNTAUTHORWWW www.novagen.com				
COMMENT	VNTAUTHORAD1 Novagen Inc.				
COMMENT	Vector_NT1_Display_Data_(Do_Not_Edit!)				
COMMENT	(SXF				
COMMENT	(CGexDoc "pET-30 Ek/LIC" 0 5439				
FEATURES	Location/Qualifiers				
promoter	complement(436..452) /vntifkey="30" /label=T7\promoter				
protein_bind	complement(344..361) /vntifkey="31" /label=His>tag				
protein_bind	complement(266..310) /vntifkey="31" /label=S>tag				
protein_bind	complement(140..157) /vntifkey="31" /label=His>tag				
terminator	complement(26..72) /vntifkey="43" /label=T7\terminator				
CDS	843..1922				

```

/vntifkey="4"
/label=lac\I
rep_origin complement(3356..3356)
/vntifkey="33"
/label=Cole1\pBR322\origin
CDS 4065..4877
/vntifkey="4"
/label=kan\sequence
rep_origin complement(4973..5428)
/vntifkey="33"
/label=f1\origin
CDS complement(409..433)
/vntifkey="4"
/label=lac\operator
protein_bind complement(317..334)
/vntifkey="31"
/label=thrombin
protein_bind complement(236..250)
/vntifkey="31"
/label=enterokinase
CDS complement(236..236)
/vntifkey="4"
/label=LIC\site
BASE COUNT 1289 a 1401 c 1464 g 1285 t
ORIGIN
1 atccggatat agttcctcct ttcagcaaaa aaccocctcaa gacccgttta gaggccccaa
61 ggggttatgc tagttattgc tcagcgggtg cagcagccaa ctcagcttcc tttcgggctt
121 tgtagcagc cggatctcag tgggtggtg ggtggtgctc gactgaggcc gcaagcttgt
181 cgacggagct cgaattcgga tccgatatcg ccatggttga ggagaagccc gggctcctgt
241 cgtcgtcatc ggtaccaga tctgggctgt ccatgtgctg gcgttcgaat ttagcagcag
301 cggtttcttt cataccagaa ccgcgtggca ccagaccaga agaatgatga tgatgatggt
361 gcatatgtat atctccttct taaagttaa caaaattatt tctagagggg aattggtatc
421 cgctcacaat tcccctatag tgagtcgtat taatttcgcg ggatcgagat cgatctcgat
481 cctctacgcc ggacgcacg tggccggcat caccggcgcc acaggtgcgg ttgctggcgc
541 ctatcgcgc gacatcaccg atggggaaga tcgggctcgc cacttcgggc tcatgagcgc
601 ttgtttcggc gtgggtatgg tggcaggccc cgtggccggg ggactggttg gcgccatctc
661 cttgcatgca ccattccttg cggcggcggg gctcaacggc ctcaacctac tactgggctg
721 cttcctaata caggagtcgc ataagggaga gcgtcgagat cccggacacc atcgaatggc
781 gcaaacctt tcgcggtatg gcatgatagc gcccggaaga gactcaattc aggggtggtg
841 atgtgaaacc agtaacgtta tacgatgtcg cagagtatgc cgggtgtctt tatcagaccg
901 tttcccgcgt ggtgaaccag gccagccacg tttctgcaa aacgcgggaa aaagtggaag

```

961 cggcgatggc ggagctgaat tacattccca accgcggtggc acaacaactg gcgggcaaac  
 1021 agtcgttgct gattggcggt gccacctcca gtctggccct gcacgcgccc tcgcaaattg  
 1081 tcgcgccgat taaatctcgc gccgatcaac tgggtgccag cgtggtggtg tcgatggtag  
 1141 aacgaagcgg cgtcgaagcc tgtaaagcgg cggtgcaaca tcttctcgcg caacgcgtca  
 1201 gtgggctgat cattaactat ccgctggatg accaggatgc cattgctgtg gaagctgctt  
 1261 gcactaatgt tccggcgta tttcttgatg tctctgacca gacacctatc aacagtatta  
 1321 ttttctccca tgaagacggt acgcgactgg gcgtggagca tctggtcgca ttgggtcacc  
 1381 agcaaatcgc gctgttagcg ggcccattaa gttctgtctc ggcgctctg cgtctggctg  
 1441 gctggcataa atatctcact cgcaatcaaa ttccagccgat agcggaaacgg gaaggcgact  
 1501 ggagtgccat gtccggtttt caacaaacca tgcaaatgct gaatgagggc atcgttccca  
 1561 ctgcatgctt ggttgccaac gatcagatgg cgctgggccc aatgcgcgcc attaccgagt  
 1621 ccgggctgcg cgttggtgcg gacatctcgg tagtgggata cgacgatacc gaagacagct  
 1681 catgttatat cccgcccgtta accacatca aacaggattt tcgcctgctg gggcaaacca  
 1741 gcgtggaccg cttgctgcaa ctctctcagg gccaggcggg gaagggcaat cagctggtgc  
 1801 ccgtctcact ggtgaaaaga aaaaccaccc tggcgcccaa tacgcaaacc gcctctcccc  
 1861 gcgcgttggc cgattcatta atgcagctgg cacgacaggt ttcccgactg gaaagcgggc  
 1921 agtgagcgcg acgcaattaa tgtaagttag ctactcatt aggcaccggg atctcgaccg  
 1981 atgcccttga gagccttcaa cccagtcagc tccttccggt gggcgccggg catgactatc  
 2041 gtcgccgcac ttatgactgt cttctttatc atgcaactcg taggacaggt gccggcagcg  
 2101 ctctgggtca ttttcggcga ggaccgcttt cgctggagcg cgacgatgat cggcctgtcg  
 2161 cttgcggtat tcggaatctt gcacgcctc gctcaagcct tcgtcactgg tcccgccacc  
 2221 aaacgtttcg gcgagaagca ggccattatc gccggcatgg cggccccacg ggtgcgcatg  
 2281 atcgtgctcc tgtcgttgag gaccgggcta ggctggcggg gttgccttac tggttagcag  
 2341 aatgaatcac cgatacgcga gcgaaactga agcagactgct gctgcaaac gtctgcgacc  
 2401 tgagcaacaa catgaatggt cttcggtttc cgtgtttcgt aaagtctgga aacgcggaag  
 2461 tcagcgcctt gcaccattat gttccggatc tgcatcgcag gatgctgctg gctaccctgt  
 2521 ggaacaccta catctgtatt aacgaagcgc tggcattgac cctgagtgat ttttctctgg  
 2581 tcccgcgcga tccataaccg cagttgttta ccctcacaac gttccagtaa ccgggcatgt  
 2641 tcatcatcag taaccggtat cgtgagcatc ctctctcgtt tcatcggtat cattaccccc  
 2701 atgaacagaa atccccctta cacggaggca tcagtgacca aacaggaaaa aaccgccctt  
 2761 aacatggccc gctttatcag aagccagaca ttaacgcttc tggagaaact caacgagctg  
 2821 gacgcggatg aacaggcaga catctgtgaa tcgcttcacg accacgctga tgagctttac  
 2881 cgcagctgcc tcgcgcggtt cggatgatgac ggtgaaaacc tctgacacat gcagctcccg  
 2941 gagacggtca cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg  
 3001 tcagcgggtg ttggcgggtg tcggggcgca gccatgacct agtcacgtag cgatagcggg  
 3061 gtgtatactg gcttaactat gcggcatcag agcagattgt actgagagtg caccatatat  
 3121 gcggtgtgaa ataccgcaca gatgcgtaag gagaaaatac cgcatcaggc gctcttccgc  
 3181 ttctctcgtc actgactcgc tgcgctcggg cgttcggctg cggcgagcgg tatcagctca  
 3241 ctcaaaggcg gtaatacggg tatccacaga atcaggggat aacgcaggaa agaacatgtg  
 3301 agcaaaaagg cagcaaaaagg ccaggaaccg taaaaaggcc gcgcttctgg cgtttttcca  
 3361 taggctccgc ccccctgacg agcatcacia aaatcgacgc tcaagtcaga ggtggcgaaa

3421 cccgacagga ctataaagat accagggcgtt tccccctgga agctccctcg tggcgtctcc  
3481 tgttccgacc ctgccgctta cgggataacct gtccgccttt ctcccttcgg gaagcgtggc  
3541 gctttctcat agctcacgct gtaggtatct cagttcgggtg taggtcgttc gctccaagct  
3601 gggctgtgtg cacgaacccc cgttcagacc cgaccgctgc gccttatccg gtaactatcg  
3661 tcttgagtcc aacccggtaa gacacgactt atcgccactg gcagcagcca ctggtaacag  
3721 gattagcaga gcgaggtatg taggcgggtgc tacagagttc ttgaagtggg ggcctaacta  
3781 cggctacact agaaggacag tatttggtat ctgcgctctg ctgaagccag ttaccttcgg  
3841 aaaaagagtt ggtagctctt gatccggcaa acaaaccacc gctggtagcg gtgggttttt  
3901 tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt  
3961 ttctacgggg tctgacgctc agtggaacga aaactcacgt taagggattt tggatcatgaa  
4021 caataaaact gtctgcttac ataaacagta atacaagggg tgttatgagc catattcaac  
4081 gggaaacgtc ttgctctagg ccgcgattaa attccaacat ggatgctgat ttatatgggt  
4141 ataaaagggc tcgagataat gtcgggcaat caggtgacgc aatctatcga ttgtatggga  
4201 agcccgatgc gccagagttg tttctgaaac atggcaaagg tagcgttgcc aatgatgtta  
4261 cagatgagat ggtcagacta aactggctga cggaaattat gcctcttccg accatcaagc  
4321 attttatccg tactcctgat gatgcatggt tactcaccac tgcgatcccc gggaaaacag  
4381 cattccaggt attagaagaa tatcctgatt caggtgaaaa tattgttgat gcgctggcag  
4441 tgttctcgcg ccggttgcat tcgattcctg tttgtaattg tccttttaac agcgatcgcg  
4501 tatttcgtct cgctcaggcg caatcacgaa tgaataacgg tttggttgat gcgagtgatt  
4561 ttgatgacga gcgtaatggc tggcctggtg aacaagtctg gaaagaaatg cataaacttt  
4621 tgccattctc accggattca gtcgctactc atgggtgattt ctacttgat aaccttattt  
4681 ttgacgaggg gaaattaata ggttgattg atgttgagc agtcggaatc gcagaccgat  
4741 accaggatct tgccatocct tggaaactgcc tcggtgagtt ttctccttca ttacagaaac  
4801 ggctttttca aaaatatggt attgataatc ctgatatgaa taaattgcag tttcatttga  
4861 tgctcgatga gtttttctaa gaattaattc atgagcggat acatatttga atgtatttag  
4921 aaaaataaac aataggggt tccgcgcaca tttccccgaa aagtgccacc tgaaattgta  
4981 aacgttaata ttttgtaaa attcgcgtta aatttttgtt aaatcagctc attttttaac  
5041 caataggccg aatcggcaa aatcccttat aaatcaaaag aatagaccga gataggggtg  
5101 agtgttggtc cagtttgaa caagagtcca ctattaaaga acgtggactc caacgtcaaa  
5161 gggcgaaaaa ccgtctatca gggcgatggc cactacgtg aaccatcacc ctaatcaagt  
5221 tttttggggg cgaggtgccg taaagcacta aatcggaacc ctaaagggag cccccgattt  
5281 agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aaggggaagaa agcgaaagga  
5341 gcggcgctca gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac cacaccgccc  
5401 gcgcttaatg cggcgctaca gggcgcgctc cattcgcca

//

(<http://www.emdbiosciences.com/docs/NDIS/69024-000.html>)

LOCUS AAB60648 320 aa linear PRI 23-AUG-2002  
 DEFINITION annexin V f.  
 ACCESSION AAB60648  
 VERSION AAB60648.1 GI:468888  
 DBSOURCE locus AH005374S01 accession U05760.1  
 locus AH005374S02 accession U05761.1  
 locus AH005374S03 accession U05762.1  
 locus AH005374S04 accession U05763.1  
 locus AH005374S05 accession U05764.1  
 locus AH005374S06 accession U05765.1  
 locus AH005374S07 accession U05766.1  
 locus AH005374S08 accession U05767.1  
 locus AH005374S09 accession U05768.1  
 locus AH005374S10 accession U05769.1  
 locus AH005374S11 accession U05770.1  
 KEYWORDS .  
 SOURCE Homo sapiens (human)  
 ORGANISM Homo sapiens  
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
 Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;  
 Catarrhini; Hominidae; Homo.  
 REFERENCE 1 (residues 1 to 320)  
 AUTHORS Cookson,B.T., Engelhardt,S., Smith,C., Bamford,H.A., Prochazka,M.  
 and Tait,J.F.  
 TITLE Organization of the human annexin V (ANX5) gene  
 JOURNAL Genomics 20 (3), 463-467 (1994)  
 PUBMED 8034319  
 REFERENCE 2 (residues 1 to 320)  
 AUTHORS Tait,J.F.  
 TITLE Direct Submission  
 JOURNAL Submitted (27-JAN-1994) Jonathan F. Tait, Department of Laboratory  
 Medicine, University of Washington, 1959 N.E. Pacific Street,  
 Seattle, WA 98195, USA  
 COMMENT Method: conceptual translation.  
 FEATURES  
 source 1..320  
 /organism="Homo sapiens"  
 /strain="Caucasian"  
 /db\_xref="taxon:9606"  
 /chromosome="4"  
 /map="4q26-q28"  
Protein 1..320  
 /product="annexin V"  
Region 20..84  
 /region\_name="Annexin"  
 /note="Annexin; pfam00191"  
 /db\_xref="CDD:40291"  
Region 91..156  
 /region\_name="Annexin"  
 /note="Annexin; pfam00191"  
 /db\_xref="CDD:40291"  
Region 173..240  
 /region\_name="Annexin"  
 /note="Annexin; pfam00191"  
 /db\_xref="CDD:40291"  
Region 249..315  
 /region\_name="Annexin"  
 /note="Annexin; pfam00191"  
 /db\_xref="CDD:40291"  
CDS 1..320  
 /gene="ANX5"

```

/coded_by="join(U05760.1:589..597,U05761.1:103..187,
U05762.1:162..256,U05763.1:109..222,U05764.1:63..153,
U05765.1:164..243,U05766.1:185..241,U05767.1:111..204,
U05768.1:99..194,U05769.1:193..251,U05769.1:479..601,
U05770.1:87..146)"
ORIGIN
  1 maqvlrgtvt dfpgfderad aetlrkamkg lgtdeesilt lltsrsnaqr qeisaafktl
 61 fgrdllddlk seltgkfecl ivalmkpsrl ydayelkhal kgagtnekv l teiiasrtpe
121 elraikqvye eeygssledd vvgdtsgyyq rmlvllqan rdpdagidea qveqdaqalf
181 qagelkwgtd eekfitifgt rsvshlrkvf dkymtisgfq ieetidrets gnleqlllav
241 vksirsipay laetlyyamk gagtdhtli rvmvrsrseid lfnirkefrk nfatslysmi
301 kgdtsgdykk allllcgedd
//

```

([http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=468888#sequence\\_468888](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=468888#sequence_468888))

## APPENDIX D

### Annexin V protein structure

Theoretical pI/Mw (average) for the user-entered sequence:

```
      10      20      30      40      50      60
MAQVLRGTVT DFPGFDERAD AETLRKAMKG LGTDEESILT LLTSRSNAQR QEISAAFKTL

      70      80      90     100     110     120
FGRDLLDDLK SELTGKFEKL IVALMKPSRL YDAYELKHAL KGAGTNEKVL TEIIASRTPE

     130     140     150     160     170     180
ELRAIKQVYE EEEYSSLEDD VVGDTSGYYQ RMLVLLQAN RDPDAGIDEA QVEQDAQALF

     190     200     210     220     230     240
QAGELKWGTD EEKFITIFGT RSVSHLRKVF DKYMTISGFQ IEETIDRETS GNLEQLLLAV

     250     260     270     280     290     300
VKSIRSIPAY LAETLYYAMK GAGTDDHTLI RVMVSRSEID LFNIRKEFRK NFATSLYSMI

     310     320
KGDTSGDYKK ALLLLCGEDD
```

Theoretical pI/Mw: 4.94 / 35936.77

([http://us.expasy.org/tools/pi\\_tool.html](http://us.expasy.org/tools/pi_tool.html))



## APPENDIX E

### EDC (Pierce Catalog)

Number	Description
--------	-------------

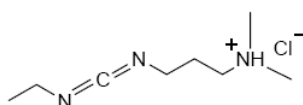
22980	EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), 5 g
-------	--

22981	EDC, 25 g
-------	-----------

77149	EDC, 10 mg
-------	------------

Molecular Weight: 191.7

CAS # 25952-53-8



**Storage:** Upon receipt store Product No. 22980 and 22981 desiccated at - 20°C. Store Product No. 77149 at 4 °C. EDC is shipped at ambient temperature.

### Introduction

EDC is a carboxyl and amine-reactive zero-length crosslinker. EDC reacts with a carboxyl group first and forms an aminereactive *O*-acylisourea intermediate that quickly reacts with an amino group to form an amide bond and release of an isourea by-product (see the Additional Information Section). The intermediate is unstable in aqueous solutions, and therefore, two-step conjugation procedures require *N*-hydroxysuccinimide for stabilization (Staros 1986; Grabarek 1990). Failure to react with an amine will result in hydrolysis of the intermediate, regeneration of the carboxyl and release of an *N*-substituted urea. A side reaction is the formation of an *N*-acylurea, which is usually restricted to carboxyls located in hydrophobic regions of proteins (Timkovich 1977; Grabarek 1990).

## Procedure for Two-step Coupling of Proteins Using EDC and NHS or Sulfo-NHS

The following protocol is adapted from a procedure described by Grabarek and Gergely (Grabarek 1990) and allows sequential coupling of two proteins without affecting the second protein's carboxyls by exposing them to EDC. This procedure requires quenching the first reaction with a thiol-containing compound.

### Materials Required

- Activation Buffer: 0.1 M MES, 0.5 M NaCl, pH 6.0
- Protein # 1: Prepared in Activation Buffer at 1 mg/ml
- Protein # 2
- NHS or Sulfo-NHS (Product No. 24500 and 24510, respectively)
- 2-Mercaptoethanol (Product No. 35600)
- (Optional) Zeba™ Desalt Spin Column (Product No. 89891) or other gel filtration column
- Hydroxylamine•HCl (Product No. 26103)

### Procedure

1. Equilibrate EDC and NHS to room temperature before opening bottles.
2. Add 0.4 mg EDC (~2 mM) and 0.6 mg of NHS or 1.1 mg of sulfo-NHS (~5 mM) to 1ml of protein #1 solution and react for 15 minutes at room temperature.
3. Add 1.4 µl of 2-mercaptoethanol (final concentration of 20 mM) to quench the EDC.
4. Optional: Separate the protein from excess reducing agent and inactivated crosslinker using a desalting column. Equilibrate the column with Activation Buffer.
5. Add protein #2 to the activated protein at an equal molar ratio with protein #1. Allow the proteins to react for 2 hours at room temperature.
6. To quench the reaction, add hydroxylamine to a final concentration of 10 mM. This method hydrolyzes nonreacted NHS present on protein #1 and results in regeneration of the original carboxyls. Other quenching methods involve adding 20-50 mM Tris, lysine,

glycine or ethanolamine; however, these primary amine-containing compounds modify carboxyls on protein #1.

7. Remove excess quenching reagent using a desalting column.