A Novel Biomedical Approach based on Artificial Cells Microencapsulated Genetically Engineered Bacterial Cells: Using *E. coli* DH5 cells for Urea and Ammonia Removal as an Example

Satya Prakash

A Thesis Submitted to the Faculty of Graduate Studies and Research,
McGill University in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Biomedical Engineering,
Faculty of Medicine,
McGill University,
Montreal, Quebec, Canada.

Artificial Cells and Organs Research Centre,
Faculty of Medicine,
McGill University,
Montreal, Quebec, Canada.

February, 1996.

ISBN 0-612-12459-2

Canada

PRAKASH / Satya

rtation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most
ly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Biomedical Engineering
SUBJECT TERM

0566
SUBJECT CODE

U·M·I

ect Categories

HUMANITIES AND SOCIAL SCIENCES

UNICATIONS AND THE ARTS
- ...cture ... 0729
- ...tory ... 0377
- ... 0900
- ... 0378
- ...ts ... 0357
- ...tion Science ... 0723
- ...ism ... 0391
- ... Science ... 0399
- ...ommunications ... 0708
- ... 0413
- ... Communication ... 0459
- ... 0465

TION
- ...l ... 0515
- ...stration ... 0514
- ...nd Continuing ... 0516
- ...tural ... 0517
- ... 0273
- ...l and Multicultural ... 0282
- ...s ... 0688
- ...nity College ... 0275
- ...lum and Instruction ... 0727
- ...hildhood ... 0518
- ...ary ... 0524
- ... 0277
- ...ce and Counseling ... 0519
- ... 0680
- ... 0745
- ...of ... 0520
- ...conomics ... 0278
- ...l ... 0521
- ...ge and Literature ... 0279
- ...iatics ... 0280
- ... 0522
- ...phy of ... 0998
- ... 0523

Psychology ... 0525
- Reading ... 0535
- Religious ... 0527
- Sciences ... 0714
- Secondary ... 0533
- Social Sciences ... 0534
- Sociology of ... 0340
- Special ... 0529
- Teacher Training ... 0530
- Technology ... 0710
- Tests and Measurements ... 0288
- Vocational ... 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General ... 0679
- Ancient ... 0289
- Linguistics ... 0290
- Modern ... 0291

Literature
- General ... 0401
- Classical ... 0294
- Comparative ... 0295
- Medieval ... 0297
- Modern ... 0298
- African ... 0316
- American ... 0591
- Asian ... 0305
- Canadian (English) ... 0352
- Canadian (French) ... 0355
- English ... 0593
- Germanic ... 0311
- Latin American ... 0312
- Middle Eastern ... 0315
- Romance ... 0313
- Slavic and East European ... 0314

PHILOSOPHY, RELIGION AND THEOLOGY
- Philosophy ... 0422

Religion
- General ... 0318
- Biblical Studies ... 0321
- Clergy ... 0319
- History of ... 0320
- Philosophy of ... 0322

- Theology ... 0469

SOCIAL SCIENCES
- American Studies ... 0323

Anthropology
- Archaeology ... 0324
- Cultural ... 0326
- Physical ... 0327

Business Administration
- General ... 0310
- Accounting ... 0272
- Banking ... 0770
- Management ... 0454
- Marketing ... 0338

- Canadian Studies ... 0385

Economics
- General ... 0501
- Agricultural ... 0503
- Commerce-Business ... 0505
- Finance ... 0508
- History ... 0509
- Labor ... 0510
- Theory ... 0511

- Folklore ... 0358
- Geography ... 0366
- Gerontology ... 0351

History
- General ... 0578
- Ancient ... 0579
- Medieval ... 0581
- Modern ... 0582
- Black ... 0328
- African ... 0331
- Asia, Australia and Oceania ... 0332
- Canadian ... 0334
- European ... 0335
- Latin American ... 0336
- Middle Eastern ... 0333
- United States ... 0337

- History of Science ... 0585
- Law ... 0398

Political Science
- General ... 0615
- International Law and Relations ... 0616
- Public Administration ... 0617

- Recreation ... 0814
- Social Work ... 0452

Sociology
- General ... 0626
- Criminology and Penology ... 0627
- Demography ... 0938
- Ethnic and Racial Studies ... 0631
- Individual and Family Studies ... 0628
- Industrial and Labor Relations ... 0629
- Public and Social Welfare ... 0630
- Social Structure and Development ... 0700
- Theory and Methods ... 0344

- Transportation ... 0709
- Urban and Regional Planning ... 0999
- Women's Studies ... 0453

SCIENCES AND ENGINEERING

ICAL SCIENCES
- ...ure
- ...eral ... 0473
- ...onomy ... 0285
- ...nal Culture and ...trition ... 0475
- ...nal Pathology ... 0476
- ... Science and ...chnology ... 0359
- ...stry and Wildlife ... 0478
- ... Culture ... 0479
- ... Pathology ... 0480
- ... Physiology ... 0817
- ...ge Management ... 0777
- ...d Technology ... 0746

- ...eral ... 0306
- ...omy ... 0287
- ...atistics ... 0308
- ...y ... 0309
- ... 0379
- ...gy ... 0329
- ...nology ... 0353
- ...tics ... 0369
- ...logy ... 0793
- ...obiology ... 0410
- ...cular ... 0307
- ...oscience ... 0317
- ...nography ... 0416
- ...ology ... 0433
- ...tion ... 0821
- ...inary Science ... 0778
- ...gy ... 0472
- ...s
- ...ral ... 0786
- ...cal ... 0760

CIENCES
- ...emistry ... 0425
- ...istry ... 0996

- Geodesy ... 0370
- Geology ... 0372
- Geophysics ... 0373
- Hydrology ... 0388
- Mineralogy ... 0411
- Paleobotany ... 0345
- Paleoecology ... 0426
- Paleontology ... 0418
- Paleozoology ... 0985
- Palynology ... 0427
- Physical Geography ... 0368
- Physical Oceanography ... 0415

HEALTH AND ENVIRONMENTAL SCIENCES
- Environmental Sciences ... 0768

Health Sciences
- General ... 0566
- Audiology ... 0300
- Chemotherapy ... 0992
- Dentistry ... 0567
- Education ... 0350
- Hospital Management ... 0769
- Human Development ... 0758
- Immunology ... 0982
- Medicine and Surgery ... 0564
- Mental Health ... 0347
- Nursing ... 0569
- Nutrition ... 0570
- Obstetrics and Gynecology ... 0380
- Occupational Health and Therapy ... 0354
- Ophthalmology ... 0381
- Pathology ... 0571
- Pharmacology ... 0419
- Pharmacy ... 0572
- Physical Therapy ... 0382
- Public Health ... 0573
- Radiology ... 0574
- Recreation ... 0575
- Speech Pathology ... 0460
- Toxicology ... 0383

- Home Economics ... 0386

PHYSICAL SCIENCES
Pure Sciences

Chemistry
- General ... 0485
- Agricultural ... 0749
- Analytical ... 0486
- Biochemistry ... 0487
- Inorganic ... 0488
- Nuclear ... 0738
- Organic ... 0490
- Pharmaceutical ... 0491
- Physical ... 0494
- Polymer ... 0495
- Radiation ... 0754

- Mathematics ... 0405

Physics
- General ... 0605
- Acoustics ... 0986
- Astronomy and Astrophysics ... 0606
- Atmospheric Science ... 0608
- Atomic ... 0748
- Electronics and Electricity ... 0607
- Elementary Particles and High Energy ... 0798
- Fluid and Plasma ... 0759
- Molecular ... 0609
- Nuclear ... 0610
- Optics ... 0752
- Radiation ... 0756
- Solid State ... 0611

- Statistics ... 0463

Applied Sciences
- Applied Mechanics ... 0346
- Computer Science ... 0984

Engineering
- General ... 0537
- Aerospace ... 0538
- Agricultural ... 0539
- Automotive ... 0540
- Biomedical ... 0541
- Chemical ... 0542
- Civil ... 0543
- Electronics and Electrical ... 0544
- Heat and Thermodynamics ... 0348
- Hydraulic ... 0545
- Industrial ... 0546
- Marine ... 0547
- Materials Science ... 0794
- Mechanical ... 0548
- Metallurgy ... 0743
- Mining ... 0551
- Nuclear ... 0552
- Packaging ... 0549
- Petroleum ... 0765
- Sanitary and Municipal ... 0554
- System Science ... 0790

- Geotechnology ... 0428
- Operations Research ... 0796
- Plastics Technology ... 0795
- Textile Technology ... 0994

PSYCHOLOGY
- General ... 0621
- Behavioral ... 0384
- Clinical ... 0622
- Developmental ... 0620
- Experimental ... 0623
- Industrial ... 0624
- Personality ... 0625
- Physiological ... 0989
- Psychobiology ... 0349
- Psychometrics ... 0632
- Social ... 0451

ation Abstracts International est organisé en catégories de sujets. Veuillez s.v.p. choisir le sujet qui décrit le mieux votre t inscrivez le code numérique approprié dans l'espace réservé ci-dessous.

U·M·I

SUJET | CODE DE SUJET

ories par sujets

LANITÉS ET SCIENCES SOCIALES

ICATIONS ET LES ARTS

- re 0729
- s 0357
- onomie 0399
- 0900
- cation verbale 0459
- cations 0708
- 0378
- e l'art 0377
- ne 0391
- 0413
- le l'information 0723
- 0465

ON

- ès 515
- ation 0514
- 0273
- ommunautaires 0275
- e 0688
- domestique 0278
- permanente 0516
- préscolaire 0518
- sanitaire 0680
- ment agricole 0517
- ment bilingue et turel 0282
- ment industriel 0521
- ment primaire. 0524
- ment professionnel 0747
- ment religieux 0527
- ment secondaire 0533
- ment spécial 0529
- ment supérieur 0745
- 0288
- 0277
- des enseignants 0530
- e l'éducation 0520
- t littérature 0279

- Lecture 0535
- Mathématiques 0280
- Musique 0522
- Orientation et consultation 0519
- Philosophie de l'éducation 0998
- Physique 0523
- Programmes d'études et enseignement 0727
- Psychologie 0525
- Sciences 0714
- Sciences sociales 0534
- Sociologie de l'éducation 0340
- Technologie 0710

LANGUE, LITTÉRATURE ET LINGUISTIQUE

- Langues
  - Généralités 0679
  - Anciennes 0289
  - Linguistique 0290
  - Modernes 0291
- Littérature
  - Généralités 0401
  - Anciennes 0294
  - Comparée 0295
  - Médiévale 0297
  - Moderne 0298
  - Africaine 0316
  - Américaine 0591
  - Anglaise 0593
  - Asiatique 0305
  - Canadienne (Anglaise) 0352
  - Canadienne (Française) 0355
  - Germanique 0311
  - Latino-américaine 0312
  - Moyen-orientale 0315
  - Romane 0313
  - Slave et est-européenne 0314

PHILOSOPHIE, RELIGION ET THÉOLOGIE

- Philosophie 0422
- Religion
  - Généralités 0318
  - Clergé 0319
  - Études bibliques 0321
  - Histoire des religions 0320
  - Philosophie de la religion 0322
- Théologie 0469

SCIENCES SOCIALES

- Anthropologie
  - Archéologie 0324
  - Culturelle 0326
  - Physique 0327
- Droit 0398
- Économie
  - Généralités 0501
  - Commerce-Affaires 0505
  - Économie agricole 0503
  - Économie du travail 0510
  - Finances 0508
  - Histoire 0509
  - Théorie 0511
- Études américaines 0323
- Études canadiennes 0385
- Études féministes 0453
- Folklore 0358
- Géographie 0366
- Gérontologie 0351
- Gestion des affaires
  - Généralités 0310
  - Administration 0454
  - Banques 0770
  - Comptabilité 0272
  - Marketing 0338
- Histoire
  - Histoire générale 0578
  - Ancienne 0579
  - Médiévale 0581
  - Moderne 0582
  - Histoire des noirs 0328
  - Africaine 0331
  - Canadienne 0334
  - États-Unis 0337
  - Européenne 0335
  - Moyen-orientale 0333
  - Latino-américaine 0336
  - Asie, Australie et Océanie 0332
- Histoire des sciences 0585
- Loisirs 0814
- Planification urbaine et régionale 0999
- Science politique
  - Généralités 0615
  - Administration publique 0617
  - Droit et relations internationales 0616
- Sociologie
  - Généralités 0626
  - Aide et bien-être social 0630
  - Criminologie et établissements pénitentiaires 0627
  - Démographie 0938
  - Études de l' individu et de la famille 0628
  - Études des relations interethniques et des relations raciales 0631
  - Structure et développement social 0700
  - Théorie et méthodes. 0344
  - Travail et relations industrielles 0629
- Transports 0709
- Travail social 0452

NCES ET INGÉNIERIE

BIOLOGIQUES

- e
- alités 0473
- omie. 0285
- ntation et technologie
- entaire 0359
- e 0479
- ge et alimentation 0475
- tation des pâturages 0777
- logie animale 0476
- logie végétale 0480
- logie végétale 0817
- ulture et faune 0478
- ologie du bois 0746

- alités 0306
- mie 0287
- ie (Statistiques) 0308
- ie moléculaire 0307
- ique 0309
- e 0379
- gie 0329
- ologie 0353
- ique 0369
- logie 0793
- biologie 0410
- ologie 0317
- nographie 0416
- ologie 0433
- tion 0821
- ce vétérinaire 0778
- gie 0472
- ue
- alités 0786
- cale 0760

DE LA TERRE

- imie 0425
- e 0996
- 0370
- ie physique 0368

- Géologie 0372
- Géophysique 0373
- Hydrologie 0388
- Minéralogie 0411
- Océanographie physique 0415
- Paléobotanique 0345
- Paléoécologie 0426
- Paléontologie 0418
- Paléozoologie 0985
- Palynologie 0427

SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT

- Économie domestique 0386
- Sciences de l'environnement 0768
- Sciences de la santé
  - Généralités 0566
  - Administration des hipitaux 0769
  - Alimentation et nutrition 0570
  - Audiologie 0300
  - Chimiothérapie 0992
  - Dentisterie 0567
  - Développement humain 0758
  - Enseignement 0350
  - Immunologie 0982
  - Loisirs 0575
  - Médecine du travail et thérapie 0354
  - Médecine et chirurgie 0564
  - Obstétrique et gynécologie 0380
  - Ophtalmologie 0381
  - Orthophonie 0460
  - Pathologie 0571
  - Pharmacie 0572
  - Pharmacologie 0419
  - Physiothérapie 0382
  - Radiologie 0574
  - Santé mentale 0347
  - Santé publique 0573
  - Soins infirmiers 0569
  - Toxicologie 0383

SCIENCES PHYSIQUES

Sciences Pures

- Chimie
  - Généralités 0485
  - Biochimie 487
  - Chimie agricole 0749
  - Chimie analytique 0486
  - Chimie minérale 0488
  - Chimie nucléaire 0738
  - Chimie organique 0490
  - Chimie pharmaceutique 0491
  - Physique 0494
  - PolymÇres 0495
  - Radiation 0754
- Mathématiques 0405
- Physique
  - Généralités 0605
  - Acoustique 0986
  - Astronomie et astrophysique 0606
  - Electronique et électricité 0607
  - Fluides et plasma 0759
  - Météorologie 0608
  - Optique 0752
  - Particules (Physique nucléaire) 0798
  - Physique atomique 0748
  - Physique de l'état solide 0611
  - Physique moléculaire 0609
  - Physique nucléaire 0610
  - Radiation 0756
- Statistiques 0463

Sciences Appliqués Et Technologie

- Informatique 0984
- Ingénierie
  - Généralités 0537
  - Agricole 0539
  - Automobile 0540
  - Biomédicale 0541
  - Chaleur et ther modynamique 0348
  - Conditionnement (Emballage) 0549
  - Génie aérospatial 0538
  - Génie chimique 0542
  - Génie civil 0543
  - Génie électronique et électrique 0544
  - Génie industriel 0546
  - Génie mécanique 0548
  - Génie nucléaire 0552
  - Ingénierie des systèmes 0790
  - Mécanique navale 0547
  - Métallurgie 0743
  - Science des matériaux 0794
  - Technique du pétrole 0765
  - Technique minière 0551
  - Techniques sanitaires et municipales 0554
  - Technologie hydraulique 0545
- Mécanique appliquée 0346
- Géotechnologie 0428
- Matières plastiques (Technologie) 0795
- Recherche opérationnelle 0796
- Textiles et tissus (Technologie) 0794

PSYCHOLOGIE

- Généralités 0621
- Personnalité 0625
- Psychobiologie 0349
- Psychologie clinique 0622
- Psychologie du comportement 0384
- Psychologie du développement 0620
- Psychologie expérimentale 0623
- Psychologie industrielle 0624
- Psychologie physiologique 0989
- Psychologie sociale 0451
- Psychométrie 0632

To my Parents

ABSTRACT

A novel approach whereby one can use genetically engineered cells for various applications is presented in this thesis. The concept of artificial cell has been used for the purpose. Artificial cells containing genetically engineered bacteria *E. coli* DH5 cells for the removal of urea and ammonia are taken as an example to establish the feasibility of this approach. Further, this example of removal of urea and ammonia is selected as presently we do not have an efficient and cost effective method to use during kidney failure, liver failure and other diseases when elevated levels of urea and ammonia are commonly experienced. In concurrence to these requirements, several in-vitro and in-vivo methods are designed and discussed in this thesis report. For these studies, alginate-polylysine-alginate microcapsules were used. Process parameters for *E. coil* DH5 cell microencapsulation, using the drop technique, have been optimized and presented. In the in-vitro studies, the batch reactor and column bioreactor have been used. Results show the possibility of using this type of artificial cells containing genetically engineered bacteria for various applications. For in-vivo experiments uremic rats were used. The details of the procedure for making uremic rat models are given. The artificial cells containing genetically engineered *E. coli* DH5 cells were administered orally to the uremic rats. Results show, only a very small dosage of artificial cells is required to lower systemic urea and ammonia. Thus, this approach can overcome problems related to the use of free bacterial cells. The efficiency of microencapsulated genetically engineered bacteria was found to be several times greater compared to standard oxystarch and urease-zirconium phosphate methods. This study has shown the primary feasibility of this new approach. The possibility of extending this approach of using artificial cells for the use of other types of genetically engineered cells for various clinical, biotechnological and biomedical applications is, therefore, potent.

RÉSUMÉ

Une nouvelle approche par laquelle des cellules programmées génétiquement peuvent être utilisées lors diverses applications est presentée dans cette thèse. Le concept de cellule artificielle est utilisé à ce propos. Des cellules artificielles enrobant les cellules bacteriénnes *E. coli* DH5, programmées génétiquement, sont prises comme exemple afin d'établir la praticabilité de cette approche. De plus l'élimination d'urée et d'ammoniaque est choisie, car présentement nous ne possédons une méthode efficace et rentable, applicable lors de l'échéances rénale, hépatique et d'autres maladies, où on rencontre très souvent des niveaux élevès d'urée et d'ammoniaque. Afin de résoudre ce problème, plusieurs méthodes in-vitro et in vivo étaient inventées et discutées dans cette thèse. Pour ces études les microcapsules alginate-polylysine-alginate étaient utilisées. Les paramètres du procédé de microencapsulation des cellules *E. coli* DH5, utilisant la technique de goutte, ont été optimisés et présentés. Pour les études in-vitro, un réacteur batch et un bioréacteur en colonne étaient utilisés. Les résultats prouvent la possibilité d'emploi de ce type de cellules artificielles contenant des cellules bactériennes programmées génétiquement dans plusieurs applications. Les expériences in-vivo s'agissaient de l'emploi des rats urémiques comme modèle animal. L'opération qui rend les rats urémiques est décrite en détail. Les microcapsules contenant les cellules bactériennes *E.coli* DH5 programmées génétiquemnt étaient administrées par la voie orale aux rats urémiques. Les résultats montrent qu'il suffit d'un petit dosage de cellules artificielles pour baisser les niveaux systémiques d'urée et d'ammoniaque. Ainsi cette approche peut surmonter les problèmes associés à l'utilisation des cellules bactériennes libres. L'efficacité des cellules programmées génétiquement microencapsulées excède plusieurs fois celle des méthodes traditionnelles d'oxystarch et d'uréase zirconium phosphate. Cette étude a demontré la praticabilité primaire de cette nouvelle approche. Ainsi, la projection de microencapsulation d'autres cellules programmées génétiquement pour diverses applications cliniques, biotechnologiques et biomédicales semble réaliste.

ACKNOWLEDGEMENTS

I wish to express my most sincere gratitude to my supervisor, Dr. Thomas Ming Swi Chang, O.C., M.D., Ph.D., F.R.C.P.(C), Director of the Artificial Cells and Organs Research Centre, Professor of Medicine, Physiology, Biomedical Engineering and Associate member of Chemistry and of Chemical Engineering. It has been an honour and a privilege to have studied under his guidance. I am very grateful to him for introducing me to the field of his pioneering artificial cell concept. I am deeply thankful for his wise counsel, patient understanding, and constant encouragement during the course of my studies here. His kindness and thoughtfulness will never be forgotten. The spiritual inspiration and order which he was able to install in me, will guide me not only in my professional life but also in my personal life.

I wish to acknowledge the Biomedical Engineering Department at McGill University. I would like to express my sincere thanks to Prof. R. Sipehia, Prof. Galiana, and Prof. P. Barre for the critical evaluation of the project and their understanding, encouragement and suggestions.

I would like to thank Miss Maryam Mobed, Mr. Colin Lister, Mr. Felice d'Angalio, Miss Vaia Coromili, Miss Pina Sorrani, Mr. Harry Wong, Dr. Lloyd-George Ian, Dr. Sylvia Bruni, Dr. T. Nishiya, and my fellow graduate students at Artificial Cells and Organs Research Centre, for their good-fellowship. Maryam Moded provided the French translation of the abstract.

I wish to acknowledge the Physiology Department, McGill University and Centre for Biochemical Technology, CSIR, New Delhi, India.

I am also grateful, truly beyond words, to my parents and family members, friends and particularly Mr. B.K. Pandey, Mr. U. Prasad, Mr. S.K. Pandey, Dr. R. Chandra, Dr. G. M. Saddi and Dr. U.S. Pandey for their unstinting encouragement and moral support.

This research has been supported by National Overseas Scholarship from the Government of India to Prakash and the Medical Research Council of Canada grant and the MESST Virage "Centre of Excellence in Biotechnology" grant to Dr. T.M.S. Chang. All these agencies are gratefully acknowledged.

PREFACE

In accordance with the guidelines concerning thesis preparation, I have taken the option of writing the experimental portion of this thesis in the form of original papers suitable for publication. This option is provided by **Section 7** in the **Guidelines Concerning Thesis Preparation**, which reads as follows:

> The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion and summary, and a thorough bibliography or reference list. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

In this thesis, manuscripts of original papers are presented in **Chapters 2 to 7**. Each paper has its own Abstract, Introduction, Materials and Methods, Results, Discussion, and References. The results which are not included in the papers are described under appendix after each paper in the concerned chapter. A common Abstract, Introduction, a final overall Conclusion, Summary, and Claims to Original Contributions are also included.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND TERMINOLOGY

APA	alginate poly-L-lysine alginate
^{0}C	degree of centigrade
dl	deciliter
E	enzyme
EC	Enzyme Committee
ESRD	End stage renal diseases
ES	enzyme-substrate complex
FAD	flavin adenine dinucleotide
G-6-P	glucose-6-phosphate
GDH	glucose dehydrogenase
GLDH	glutamic dehydrogenase
g/L	gram/liter
gm	gram
hr	hour
LDH	lactate dehydrogenase
LEUDH	leucine dehydrogenase
M	molar
m	meter
m2	square meter
MDH	malate dehydrogenase
mg	milligram
min	minute
ml	milliliter
mM	millimolar
M.W.	molecular weight
N	normal
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometer
No.	number
P	product
P.B.	phosphate buffer
PEG	polyethylene glycol
PEI	polyethylenimine

PL	Polylysine
pp	pages
rpm	revolutions per minute
S	substrate
SD	standard deviation
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
U.Z.P.	urease zirconium phosphate
v	velocity
>	greater than
<	less than
µl	microliter
µM	micromolar
µmol	micromole

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The existing medical care technology has achieved a great deal of success. In the 19th century, the planet Earth could sustain only 1 billion people. Ten thousand years ago, only 4 million could keep themselves alive. Now, more than 5 billion people are living longer and healthier than ever before, on average. We have seen extraordinary progress for the human accomplishment, especially in the past two centuries. The most important and amazing fact--the greatest human achievement in history is the decrease in the world's death rate. It took thousands of years to increase life expectancy at birth from just over 20 years to the high 20s. Then in just the past two centuries, the length of life one could expect for a new-born in the advanced countries jumped from less than 30 years to perhaps 75 years. Starting in the1950s, well after World War II, length of life in the poor countries leaped upward by perhaps 15 or even 20 years (Statistical abstract of the United States, 1990, p. 75; WHO world health report, 1995). This has been possible only because of a big contribution from modern medical research and practice.

The question, however, is whether we are well equipped to maintain our superiority over diseases ? or we are in a state of crisis ? whether we will be able to provide an affordable health care to everyone by lowering the involved cost or not ? The answer is simple. We have not achieved a complete control over diseases and nor in a state of crisis. However, reports about the emergence of new diseases, the fresh outbreak of old diseases, for example, tuberculosis, and the emergence of antibiotic resistant pathogens is certainly alarming. We, therefore, need to look for new tools, new technologies, and a better art to practice medicine to meet the present need and future challenges as well as significant cost savings.

The discovery of the techniques of genetic manipulation certainly has enhanced our power to fight to solve present problems and a big hope to manage future healthcare better. Genetically engineered smart drugs and enzymes are already in use. Cell therapy, gene therapy, tissue engineering are the few other examples of molecular biology based present

day approaches. Similarly, the concept of artificial cells by Chang (Chang, 1964,1995) is another powerful technology that may help to meet both present and future challenges. This thesis pertains to venture the combine use of these two powerful technologies, namely recombinant DNA technology and artificial cells. If successful, this research will open a new approach of using artificial cells containing genetically engineered bacterial cells for disease control, as with the exploding rise in molecular biology research a variety of genetically engineered cells with many specialised functions are increasingly available. In this thesis, artificial cells containing genetically engineered bacteria *E. coil* DH5 cells for the removal of urea and ammonia is taken as an example for a feasibility study.

Each year millions of people around the globe, and more than 50,000 people in the United states alone, are diagnosed with end stage renal diseases (ESRD), a serious condition in which the kidney fails. When both of our kidneys fail, the body holds fluid and the blood pressure rises. Harmful waste builds up in our body. In uremia there is a high level of uremic wastes and toxins in the blood and other body fluid compartments. It is necessary to lower their concentrations before they cause serious damage to the patients.

A variety of methodologies, such as transplantation, dialysis, hemofiltration, hemoperfusions, etc., are available for the removal of unwanted metabolites in uremia. These methodologies, however, are very expensive. For instance, the cost for care of patients with ESRD in USA alone was $7.26 billion in 1990 (NIH Census Statement,1993). Further, none of these methodologies offer a complete solution to the problem. For example, except for dialytic type of treatment presently we do not have a simple and effective method for the removal of urea and ammonia from the body fluid compartments during kidney failure, liver failure and other diseases. Several approaches has been used in the past for urea and ammonia removal. These can be broadly divided into five groups: i) Biological approaches: organ transplantation, etc., (Ganong, 1995; WHO report, 1995), ii) Physical approach: use of dialysis, etc. (Friedman, 1972,1974, 1995; Sparks, 1972, 1974), iii) Chemical approach: this includes the use of oral ingestion of adsorbents that bind urea and ammonia in the intestine, e.g. oxystarch, zirconium phosphate etc.(Friedman, 1972,1973; Sparks, 1972), and iv) Enzymatic approach: one example is the use of micoencapsulated urease that converts urea into ammonia which is then removed by

adsorbent (Chang, 1966,1976b,1984; Cataneo and Chang, 1984). The other example is the use of microencapsulated multienzyme systems that convert urea and ammonia into essential amino acids (Gu and Chang,1991,1992) and v) Colonisation of the intestinal tract with microorganisms which can remove urea and ammonia (Setala, 1971; Setala et al., 1972,1973).

All these approaches are associated with a number of problems. For example, the involvement of complex surgery, lack of suitable donors, rejection, and side effects of the immunosuppresant drugs used are a few of the problems associated with transplantation. The existing dialysis treatment is complex and expensive. Hemoperfusion does not remove urea and ammonia. High dosages are required for oxystarch and urease-zirconium phosphate. The conversion rate is very low for the multienzyme complex and urea utilising soil bacteria. These are some of the many problems associated with presently available systems and past approaches. Therefore, it is urgent to look for a new approach for urea and ammonia removal. In brief, therefore, this thesis investigates the following approaches:

(1) A general approach to establish the use of artificial cells containing genetically engineered bacterial cells for various applications, and

(2) A specific approach which will explore specific use of artificial cells containing genetically engineered bacteria *E. coli* DH5 cells for the removal of urea and ammonia for its potential use in kidney failure, liver failure and other diseases.

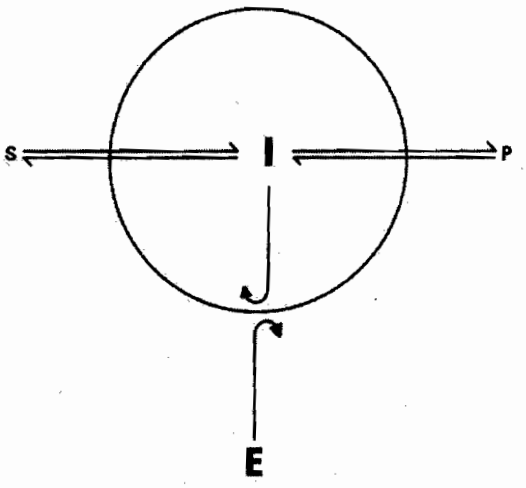
These two approaches are novel. They demonstrate the first successful use of artificial cells containing genetically engineered bacterial cells for the direct biomedical application in general and for the removal of urea and ammonia using artificial cells containing genetically engineered bacteria *E. coli* DH5 in particular. The concept investigated in this thesis, therefore, if successful, may establish a precedent for potential use of artificial cells containing this and other types of genetically engineered microorganisms for various applications.

In the following sections I will review the relevant literature to date. The concise statement of the objectives of the thesis are summarised at the end.

1.2 ARTIFICIAL CELLS

1.2.1 The Concept

The concept and feasibility of artificial cells was first shown by Chang (Chang,1957, 1964). He utilises some of the properties of biological cells for making artificial cells. Like all living cells, each artificial cell consists of a selectively permeable membrane of cellular dimensions, enveloping biologically active materials. The membrane of each artificial cell, therefore, selectively separates its content (I) from the external environment (E). Permeant molecules (s,p) can diffuse rapidly in and out of the artificial cells. A schematic representation of artificial cell concept is shown in figure 1.1.



I : Intracellular material
E: Extracellular impermeant materials
S,P: Permeant molecules.

Figure 1.1: A schematic illustration of concept of the artificial cell (*Source: Chang, 1972*).

1.2.2 Methods for Preparing Artificial Cells

Cells are the structural and functional units of all living organisms. They vary, however, enormously in size, shape, and specialised functions (Lehninger, 1982). In 1957 Chang first demonstrated the feasibility of making artificial cells. "Artificial cell" as he emphasises is not a specific physical entity, "It is an idea involving the preparation of artificial structures of cellular dimensions for various possible biomedical applications" (Chang, 1972a). Since then he and many groups have developed and extended this idea of artificial cells (Agishi et al., 1980; Chang, 1969a, 1972a, 1976a,1972a, 1976b, 1977, 1978, 1980a, 1994,1995; Chang and Malave, 1970; Chang et al., 1971, 1972; Chang and Migchelsen, 1973; Gelfand et al., 1978; Okada et al., 1978; Lim and Sun, 1980; Sefton and Nishimura,1980; Goosen, 1988; Williams and Murray-Lyon, 1975; Chang and Geyer, 1989; Hunt and Burnette, 1983; Keipert and Chang, 1983, 1984; Wong and Chang, 1993; others).

1.2.3 Basic Features of Artificial Cells

1.2.3.1 Membrane Compositions and Configurations

Each artificial cell has a spherical ultrathin selectively permeable membrane. Chang made the first artificial cells by interfacial precipitation of cellulose nitrate (Chang, 1957). Since then, about 30 different polymer membranes and several protein membranes have been created, such as nylon membranes, complex lipid membranes, cross linked protein membrane and silicone rubber membranes (Chang, 1966, 1972a, 1977). Numerous chemical reactions are presently being used for making artificial cells by other interfacial polymerisation methods (Chang, 1964, 1972a; Chang et al., 1966;

Mori et al., 1973; Shiba et al., 1970; Aisina et al.,1976; Grunwald and Chang, 1981). Chang also developed multiple-compartmental membrane systems to envelop small artificial cells within larger artificial cells (Chang, 1965, 1972a). A similar double-emulsion approach was evolved using a special hydrocarbon liquid to form a liquid- surfactant membrane (May and Li, 1972, 1977).

Biological and biodegradable membranes are also available for encapsulating cells, enzymes and other biologically active materials. These membranes include: cross-linked protein membranes (Chang, 1964,1972a, 1977; Chang et al., 1966), heparin-complexed polymer membranes (Chang et al., 1967), lipid membranes (Mueller and Rudin, 1968), lipid-polymer membranes (Chang, 1969b, 1972a; Ilan and Chang, 1986; Rosenthal and Chang, 1980; Yu and Chang, 1982), liposomes (Bangham et al., 1974; Gregoriadis, 1984; Gregoriadis and Allison, 1980; Mobed and Chang, 1991,1993), and erythrocytes (Desnick et al., 1977; Ihler et al., 1973). Biodegradable polymer membranes, such as polylactic acid membrane or polyglycolic acid membrane, have also been used to encapsulate cells, enzymes or hormones (Chang, 1976c; Makino et al., 1985). Other approaches include liquid membrane (May and Li, 1972, 1977), fibre extrusion (Dinelli, 1972; Dinelli et al., 1976), and hollow-fibers (Chambers et al., 1974; Ishikawa et al., 1989; Kitano and Ise, 1984; Rony, 1971; Goosen, 1995).

1.2.3.2 Properties of the Membranes

In systems employing artificial cells, the selectively permeable membrane prevents encapsulated materials from leaking out (Chang, 1964, 1965, 1972). Thus, artificial cells immunoisolate the encapsulated materials and hence reduce immunological problems arising in the host. However, small permeant molecules such as urea and ammonia can equilibrate rapidly across the membrane and contact the encapsulated materials. Furthermore, proteolytic enzymes existing in the surrounding

environment cannot cross the membrane to deactivate the encapsulated materials.

The artificial cell's membrane provides a large surface area to volume ratio. Ten millilitres of 20 μm diameter artificial cells have a total surface area of 2.5 m^2 (Chang, 1966). This area is more than twice that of the membrane present in standard hemodialysis machines (1 m^2). Furthermore, each artificial cell has an ultrathin membrane of 20 nm thickness, which is at least 100 times thinner than that of the standard hemodialysis machines (Chang, 1966,1972a; Chang et al., 1966). Therefore, artificial cells allow external permeant molecules to equilibrate much faster than in standard hemodialysis machines.

1.2.4 Contents within Artificial Cells

Theoretically, an artificial cell can contain virtually anything: drugs, enzymes, antibodies, cell extracts, cells, etc. (Chang, 1957,1964,1972,1985, 1994,1995; Leigh Dayton, 1989). Some of the materials encapsulated are enzymes, proteins, and cell extracts (Bourget and Chang, 1986; Chang, 1964, 1965, 1972a, 1977; Chang and Poznansky, 1968; Garofallo and Chang, 1989; Lloyd-George and Chang, 1993; Kitajima and Kondo, 1971; Mosbach and Mosbach, 1966; Ostergaard and Martiny, 1973; Shi and Chang, 1982; Shu and Chang, 1980, 1981; Siu Chong and Chang, 1974; Yuan and Chang, 1986). Multienzyme systems, and a combination of enzymes and adsorbents, have also been encapsulated (Chang, 1964, 1966; Gardner et al., 1971; Sparks et al., 1971, Gu and Chang,1992). Enzymes have also been encapsulated together with a magnetic material (Chang, 1966, 1977). Other materials which have been encapsulated include radioisotope-labelled materials (Chang, 1977; Chang et al., 1966), insolubilized enzymes (Chang, 1969c, 1972a), coenzyme recycling multienzyme systems (Campbell and Chang, 1975, 1976; Chang and Malouf, 1978, 1979; Chang et al., 1979; Cousineau and Chang, 1977; Grunwald and Chang, 1979, 1981; Yu and Chang, 1982), antigens, antibodies, vaccines, hormones, and pharmaceuticals (Chang, 1976c, 1977; McGinity and Cuff,

1985).

Microencapsulation of living cells and microorganisms have also been developed (Chang, 1965, 1972a; Chang et al., 1966; Mosbach and Mosbach, 1966; Garofallo and Chang, 1984; Lloyd-George and Chang, 1993). This procedure was proposed for use in the encapsulation of islets for the treatment of diabetes and hepatocytes for the treatment of liver failure (Chang, 1965, 1972a; Chang et al., 1966). Lim and Sun (1980) developed this and microencapsulated rat islet cells, which were then implanted intraperitoneally into the diabetic rats. These encapsulated islet cells were able to maintain normal glucose levels in the diabetic animals for up to one year. Artificial cells containing fibroblasts or hybridomas have also been used in biotechnology for the production of interferon and monoclonal antibodies (Damon Corporation, 1981). Ergan et al. (1984) immobilized a bacterium containing NADH-oxidase by microencapsulation. Rat hepatocytes were also encapsulated within alginate-polylysine membranes and served as a liver support system (Bruni and Chang, 1995; Cai et al., 1988; Chang, 1995; Wong and Chang, 1986, 1988). Microencapsulation of other mammalian cells, such as human erythrocytes and human diploid fibroblasts, have also been accomplished (Boag and Sefton, 1987; Sefton and Broughton, 1982; Stevenson et al., 1988; Sugamori et al., 1985).

1.2.4.1 Artificial Cells for Living Cell and Tissue Immobilization

The term "immobilized enzyme and cell" was adopted by an international committee at the First Enzyme Engineering Conference (Sundaram et al., 1972). There are many reasons for the immobilization of enzymes and cells. The main advantages of immobilization are: (a) it offers operational advantages over its freely mobile counterparts, (b) it may exhibit selectively altered chemical or physical properties, and (c) it may serve as a model system for natural or in-vivo system (Chibata, 1984). Not all of the immobilization methods available can achieve these aims. However, the general operational advantages of immobilized cell and enzymes are reusability, stability, and

convenience.

Cells and enzymes can be immobilized by :

(a) adsorbing them onto the surfaces of substances such as kaolin or woodchips or PTFE;

(b) cross linking them into pellets with bifunctional agents such as gluteraldehyde;

(c) entrapping them within a gel or matrix ; and

(d) entrapping them inside a physical barrier, such as a semipermeable membrane of microcapsules of artificial cells.

Microencapsulation is one of the most convenient barrier methods available for complete viable cell immobilization. Chang (1965,1972) first showed the feasibility of cell encapsulation and proposed its use for cell transplantation. This has been developed for the encapsulation of viable cells such as hepatocytes or islets. Membrane prepared from cross-linked protein (Chang ,1965,1972; Chang et al., 1966), alginate-polylysine (Lim and Sun, 1980; Lim, 1984), chitosan-alginate (McKnight et al., 1988), cellulosesulphate-poly(dimethyldiallyl)-ammonium chloride (Stefuca et al., 1991), hydroxyethyl methacrylate-methyl methacrylate (Stevenson et al., 1988), and chitosan-carboxymethyl-cellulose (Yoshioka et al.,1990) have been used as membrane forming compounds.

Presently the reaction between polylysine and alginate to produce a membrane is the most popular for cell encapsulation. This method is simple and no harsh solvents are required. Briefly, the cells are suspended in a sodium alginate solution, droplets of this cell suspension are then gelled by contact with ice cold calcium chloride solution. The calcium alginate beads with entrapped cells are then coated with a polylysine solution. The polycationic polylysine interacts with the polyanionic alginate to form a polylysine-alginate membrane. A further coat with a low concentration alginate solution is usually applied. This results in the formation of an alginate-poly-L-lysine-alginate membrane around a calcium alginate infra-structure. The interior of the bead is then liquefied by treating the beads with a sodium citrate solution. The citrate sequesters the calcium from the gel and the gel is then liquified. The calcium citrate is removed by washing with

saline (Lim and Sun, 1980; Lim, 1984; Lim, 1988). This process and the optimization of the process will be described in more detail in chapter 3 of this thesis.

Microcapsules made by this process have been used to encapsulate islets which have been used in the treatment of experimental diabetes in rats (Lim and Sun, 1980; Lim, 1984, 1988). Microencapsulated hepatocytes for the treatment of experimental liver failure in rats (Bruni and Chang, 1995; Wong and Chang, 1986; Sun et al., 1986) was mentioned earlier.

Alginate-poly-lysine-alginate microcapsules can also be used for the culturing of hybridomas in bioreactors. A small concentration of hybridomas are encapsulated. The hybridomas multiply and produce monoclonal antibodies (MoAb). The molecular weight of the monoclonal antibody is larger than the molecular weight cut off of the membrane and so it is trapped inside the microcapsule. The microcapsules can be easily separated from the culture medium and then broken to release the entrapped protein. This process for making monoclonal antibodies is cheaper since the MoAb is easily separated from the media constituents. The MoAb from the microcapsules is also partially purified and concentrated.

Several improvements to the basic alginate-polylysine-alginate microencapsulation procedure have been made. Goosen *et al.* (1980) examined the effect of molecular weight of the polylysine on the molecular weight "cut-off" of microcapsules and found that the low molecular weight PLL increased the strength and reduced the permeability of the microcapsule membrane. It was felt that low molecular weight PLL could penetrate into the alginate bead and cross-link the alginate and produce a more compact membrane (Goosen et al.,1985; King et al., 1987). The polylysine coating step can be done in 2 stages. In the first stage a high molecular weight polylysine is used at the initial membrane forming stage. This leads to microcapsules that are very permeable. Therefore, in the internal liquefaction stage some of the alginate within the microcapsules will leak out. The microcapsules are then treated with a low molecular weight PLL to reinforce the membrane of the microcapsules. When hybridomas were cultured in these microcapsules they accumulated more MoAb protein (King et al., 1987; Goosen et al., 1989).

The biocompatibilty of poly-L-lysine-alginate microcapsules is improved when an additional coat of alginate is applied as described above (Clayton et al., 1991,1992; Soon-Shiong et al., 1991). This improvement is due to the fact that the positively charged PLL is immunogenic, while the combination of alginate and PLL is neutral and thus less immunogenic. Alginate is a linear polysaccharide composed of 1,4 linked P-D-mannuronic acid (M) and (X-L-guluronic acid (G) units (Soon-Shiong et al., 1991). It was found in one study that a final coat with a high G alginate caused less fibrosis around the microcapsules (Soon-Shiong et al., 1991). In another study different researchers reported that a final coat with a high M alginate would reduce the amount of fibrosis around peritoneally implanted microcapsules (Clayton et al., 1991,1992). A microcapsule membrane infiltrated with cells will be discontinuous and could consequently be weak (Wong and Chang, 1989). If these cells protrude from the microcapsule they could trigger the immunological rejection of implanted microcapsules (Clayton et al., 1991,1992; Wong and Chang, 1988a).

In order to further improve their biocompatibility and prevent cell protrusion from the membrane a "two-step" procedure for making alginate-polylysine-alginate microcapsules was developed by Wong and Chang (Wong and Chang, 1988b). Calcium alginate beads with entrapped hepatocytes were suspended in a 1.5 % sodium alginate solution. This suspension was then extruded as droplets which were then microencapsulated. In the internal liquefaction step the beads within the microcapsule are also liquefied. This "two-step" procedure then produces microcapsules with cells inside, however there are no cells in the membrane of these microcapsules (Wong and Chang, 1991). Wong and Chang have shown that there is less fibrosis around intra-peritoneally implanted microcapsules, if they are made by the two-step procedure, as opposed to those made by the traditional approach (Wong and Chang, 1991).

The consequence of subtle differences in the preparation of alginate-poly-L-lysine-alginate microcapsules were underlined in a report by Goosen *et al,* who mentioned that the permeability of the microcapsules to a protein could vary from batch to batch, even if the "same" conditions were used in the preparation of the microcapsules to adjust permeability characterization of microcapsules (King et al., 1987). Coromili and

Chang, using the HPLC, analyzed the mass transfer of dextran with a large spectra of different molecular weights (Coromili and Chang, 1993; King et al., 1987). This result on permeability, and the conflicting reports on the role of high G or high M alginates on biocompatibility, show that the conditions during microencapsulation must be carefully controlled. If this is not done it is difficult to determine the exact effect of changes in process variables.

The variation in the biocompatibilty of different types of alginate-poly-L-lysine-alginate microcapsules partially explains the great variation in the results obtained when they are intra-peritoneally implanted. For instance some continue to work for only few weeks, others for months to years (Wong and Chang, 1986; Bruni and Chang, 1989; Cai et al., 1989; Klein et al., 1983; Chang, 1990).

Many excellent books and reviews are available on cell and enzyme immobilization (Blanch and Klibanov, 1988; Broun et al., 1978; Chang, 1972a, 1977, 1988,1990,1995; Chibata, 1978; Chibata et al., 1982, 1983; Goldman et al., 1971; Kennedy, 1987; Laskin et al., 1984, 1987; Manecke, 1974; Messing, 1975; Moo-Young, 1985; Mosbach, 1976, 1987, 1988; Neufiled et al., 1995; Pye and Weetall, 1978; Pye and Wingard, 1974; Scouten, 1983; Vieth, 1979, 1988; Weetall and Royer, 1980; Wingard, 1972; Wingard et al., 1976, 1979, 1980, 1981; Zaborsky, 1973).

1.2.5 Biomedical Application of Artificial Cells

Artificial cells containing enzymes have been used in a number of experimental and therapeutic conditions (Chang, 1972a, 1977, 1984b, 1988, 1994,1995). The use of artificial cells containing urease is the first model of immobilized enzymes for experimental therapy (Chang, 1964,1965,1966, 1972a). Ten millilitres of microencapsulated urease in an extracorporeal hemoperfusion chamber lowered the systemic blood urea of dogs by 50% within 45 minutes (Chang, 1966). The ammonia formed was removed by a microencapsulated ammonia adsorbent (Chang, 1966). Oral

administration of these artificial cells has been found to remove the urea in the intestinal tract of rats (Chang and Loa, 1970; Gardner et al., 1971). This approach has developed to a stage for clinical assessment (Kjellstrand et al., 1981). These applications of microencapsulated urease have paved the way for other types of enzyme replacement therapies.

Chang and Poznansky (1968) prepared artificial cells containing the enzyme catalase by a phase separation method, using collodion. They studied the effects of the intraperitoneal administration of catalase into mice with a hereditary catalase deficiency, i.e. acatalasemic mice. The results showed that catalase administrated either in free solution or in artificial cells is efficient in replacing the function of the deficient catalase enzyme. Repeated injection of catalase in solution results in the production of antibodies. These immunized acatalasemic mice suffer hypersensitivity reactions to further catalase injections. On the other hand, the repeated injections of catalase-loaded artificial cells do not induce catalase antibody formation in mice and therefore successfully prevent the hypersensitivity reactions to catalase (Poznansky and Chang, 1974). These results support the proposed use of artificial cells for replacement therapy in congenital enzyme deficiency diseases. Bourget and Chang (1986) later developed artificial cells containing phenylalanine ammonia-lyase for the depletion of systemic L-phenylalanine in phenylketonuric rats (Bourget and Chang, 1986).

Enzyme L-Asparaginase has also been microencapsulated into artificial cells (Chang, 1969d, 1971, 1973; Mori et al., 1972, 1973). Chang studied the effects of administering microencapsulated L-asparaginase to mice with substrate- dependent tumors (Chang, 1971; Siu Chong and Chang, 1974). The microencapsulated L-asparaginase suppresses the growth of implanted lymphosarcoma cells more effectively than L-asparaginase given in free solution form (Chang, 1971). Another example of the biomedical uses of artificial cells is the use of microencapsulated tyrosinase to remove L-tyrosine in a perfusion system (Fischer, 1975). It is also used to remove galactosamine-induced fulminant hepatic failure rats which have high levels of L-tyrosine (Shi and Chang, 1982; Shu and Chang, 1980, 1981).

A summary of these and other biomedical and biotechnological application of artificial cells is given below (Table 1.1).

1. Treatment of acute poisoning (routine clinical application)
2. Treatment of aluminum and iron overload (routine clinical application)
3. Treatment of end-stage kidney failure (routine clinical application as a supplement to hemodialysis)
4. Treatment of liver failure (routine clinical application for certain types of acute liver failure)
5. Red blood cell substitutes for transfusion (clinical trial started)
6. Blood group antibody removal (clinical application and clinical trial)
7. Treatment of hereditary enzyme deficiency (clinical trial started)
8. Clinical laboratory analysis (clinical application)
9. Production of monoclonal antibodies (routine use in industry)
10. Treatment of diabetic mellitus and other endocrine diseases (animal experiment)
11. Drug delivery systems (clinical application in some cases)
12. Conversion of cholesterol into carbon dioxide (experimental for dairy products and blood cholesterol)
13. Bilirubin removal (experimental)
14. Production of fine biochemicals (industrial application)
15. Food and aquatic culture (industrial application)
16. Conversion of wastes into useful products (experimental)
17. Other biotechnological and medical applications (in progress)

Table 1.1: A summary of principle biomedical and biotechnological application of artificial cells. *Source: Chang; Encyclopaedia of Human Biology, 1991 .*

Artificial cells containing encapsulated cells and tissues were also used for biotechnological, food technological, biomedical applications. Similarly many excellent reviews for the biotechnology and biomedical applications are available (Chang, 1972, 1985, 1990, 1992, 1995; Goosen et al., 1988; Hoffman et al., 1986; Klien et al., 1985). A recent review by Groboillot et al. (1995) summarises the details of food industry application of microencapsulation.

1.3 UREA REMOVAL IN RENAL FAILURE

1.3.1 Introduction

Normal renal functions include: removal of the end products of metabolism including urea, uric acid, creatinine, ammonia, and others; regulation of the body chemistry : regulation of water and electrolytes; and excretion of some drugs and toxins. The other function is the production and secretion of enzymes and hormones, for example, renine, erythropoietin, and 1, 25-Dihydroxyvitamin D3, the biologically most active form of the vitamin D3, is formed in tubule cells (Guyton, 1995).

When normal function of the kidney stops or deteriorates due to disease, the patient become progressively ill. Metabolic waste products, such as urea, uric acid, creatinine, etc., excess electrolytes and water accumulates in the body with resulting chemical balance upset followed later by death (Oberley et al., 1991;Guyton, 1995). There are many procedures that can be used to maintain the life of uremic patients, for example, kidney transplantation, dialysis, hemofiltration, etc.

1.3.2 Kidney Transplantation

Kidney transplantation is a procedure that places a healthy kidney from another person into the renal failure patients body (Oberley et al., 1991; Guyton, 1995). There is always a chance that recipient body will reject the new kidney, despite the use of immunosuppressants, no matter how good the match. The chance of accepting the new kidney depends on age, race, and medical condition (Ganong, 1995). Normally, 75 to 80 percent of transplants from cadaver donors are working one year after surgery (US Kidney foundation report, 1995). However, transplants from living relatives often work

better than transplants from cadaver donors. This fact is because they are usually a closer match (Ganong, 1995). Immunosuppressive drugs that help to prevent rejection are used. Treatment with these drugs, however, may cause side effects (Ganong, 1995).

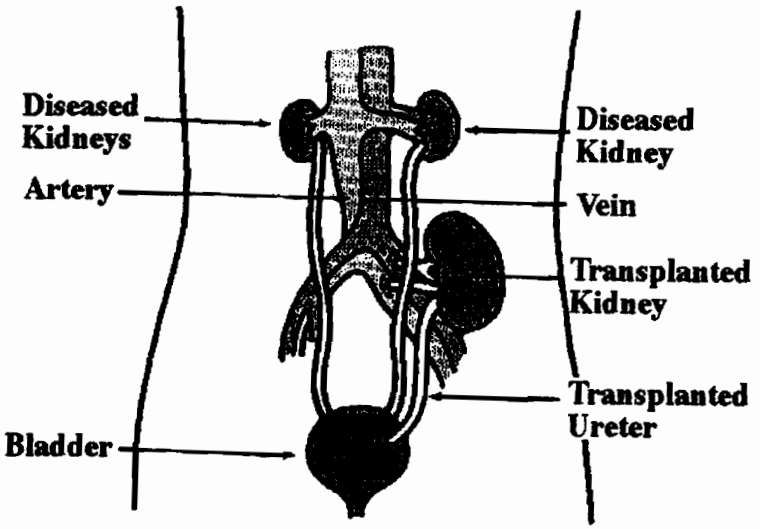


Figure 1.2 A schematic presentation of kidney transplantation procedure.

There are pros and cons to kidney transplantation. The pros of kidney transplantation is that : it works like a normal kidney; it helps patients feel healthier; may not need to go for diet restrictions and there's often no need for other supportive treatment. The cons include : major surgery requirements; long waiting periods for a suitable donor; possible rejection and the need to take drugs for the rest of his life.

1.3.3 Dialysis

1.3.3.1 Hemodialysis

Hemodialysis is a procedure that cleans and filters blood. It rids renal failure patients body of uremic wastes, extra salt, and fluids. It controls blood pressure and helps to keep the proper balance of water and electrolytes such as potassium, sodium, and chloride. Hemodialysis uses a dialyzer to clean the blood. During the treatment, an access to the bloodstream must be made to provide a way for blood to be carried from the body to the dialysis machine and then back into the body (Oberley et al., 1991; Guyton, 1995).

Hemodialysis can be done at home or at a dialysis center. Hemodialysis usually is done three times a week (Oberley et al., 1991). Side effects can be caused by rapid changes in the patients body fluids and chemical balance during treatment (Oberley et al., 1991). Muscle cramps and hypertension are the two common reported side effects (Oberley et al., 1991).

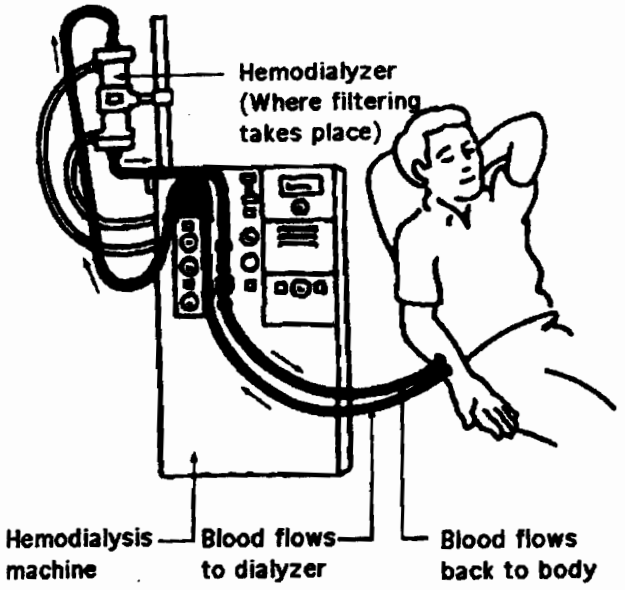


Figure 1.3: A schematic illustration of an ongoing hemodialysis process.

There are a number of cons associated with hemodialysis. Support of trained professionals is required. It is also an expensive, complex and bulky system (Oberley et al., 1991).

1.3.3.2 Peritoneal dialysis

Peritoneal dialysis removes extra water, wastes, and chemicals from the body. This type of dialysis uses the peritoneal lining for dialysis and ultrafiltration. This lining is the peritoneal membrane. A solution, called dialysate, travels through a special tube into the peritoneal cavity. Fluids, wastes, and chemicals pass from capillaries of the peritoneal membrane into the dialysate. After several hours, the dialysate gets drained from the abdomen, taking the wastes from blood with it. The abdomen is refilled with fresh dialysate and the whole cleaning process can be repeated (Oberley et al., 1991).

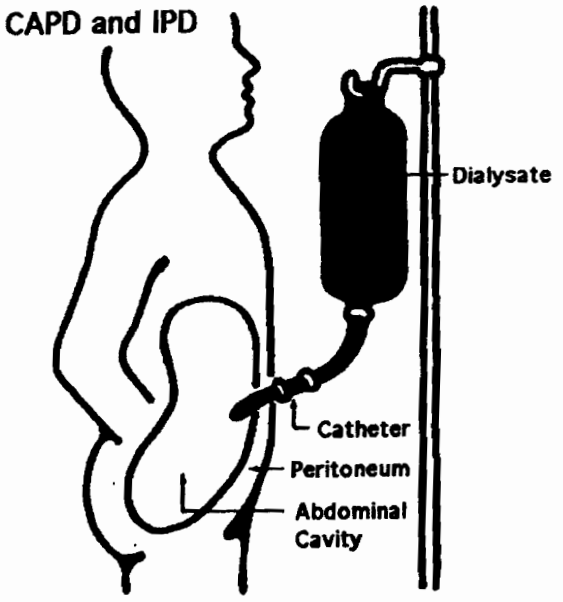


Figure 1.4: A schematic illustration of an ongoing peritoneal dialysis process.

There are three types of peritoneal dialysis.

1. Continuous Ambulatory Peritoneal Dialysis (CAPD):

CAPD is the most common type of peritoneal dialysis. It needs no machine. It can be done in any clean, well-lit place. With CAPD, the blood is always being cleaned. The dialysate passes from a plastic bag through the catheter and into the abdomen. The dialysate stays in the abdomen with the catheter sealed. The patient is completely ambulatory during this time. After several hours, it is required to drain the solution back into the bag. A refill of the peritoneal cavity with fresh solution through the same catheter is required to begin the cleaning process again. While the solution is in the body, the patients may fold the empty plastic bag and hide it under the clothes, around the waist, or in a pocket.

2. Continuous Cyclic Peritoneal Dialysis (CCPD):

CCPD is like CAPD except that a machine, which connects to the catheter, automatically fills and drains the dialysate from the abdomen. The machine does this at night while patients sleep.

3. Intermittent Peritoneal Dialysis (IPD):

IPD uses the same type of machine as CCPD to add and drain the dialysate. IPD can be done at home, but it is usually done in the hospital. IPD treatments take longer than CCPD.

CAPD is a form of self-treatment. It needs no machine and no partner. However, with IPD and CCPD, we need a machine and the help of a partner (family member, friend, or health professional). With CAPD, the dialysate stays in the abdomen for about 4 hours. The process of draining the dialysate and replacing fresh solution takes 30 to 40

minutes. Most people change the solution four times a day (Oberley et al., 1991; Briggs and Schnermann, 1994). With CCPD, treatments last from 10 to 12 hours every night. With IPD, treatments are done several times a week, for a total of 36 to 42 hours per week. Sessions may last up to 24 hours (Oberley et al., 1991; Briggs and Schnermann, 1994).

Mechanical complications such as pain, intra-abdominal bleeding; infections and inflammatory complications; cardiovascular complications; pulmonary complications and neurological complications are the most often mentioned complications with peritoneal dialysis (Oberley et al., 1991; Briggs and Schnermann, 1994). Peritonitis can occur, if the opening where the catheter enters your body gets infected (Briggs and Schnermann, 1994). The diet for peritoneal dialysis is slightly different than the diet for hemodialysis. More salt and fluids and protein are allowed. However, potassium restrictions and a reduced calorie intake are required. This limitation is because the sugar in the dialysate may result in weight gain (Oberley et al., 1991; Briggs and Schnermann, 1994).

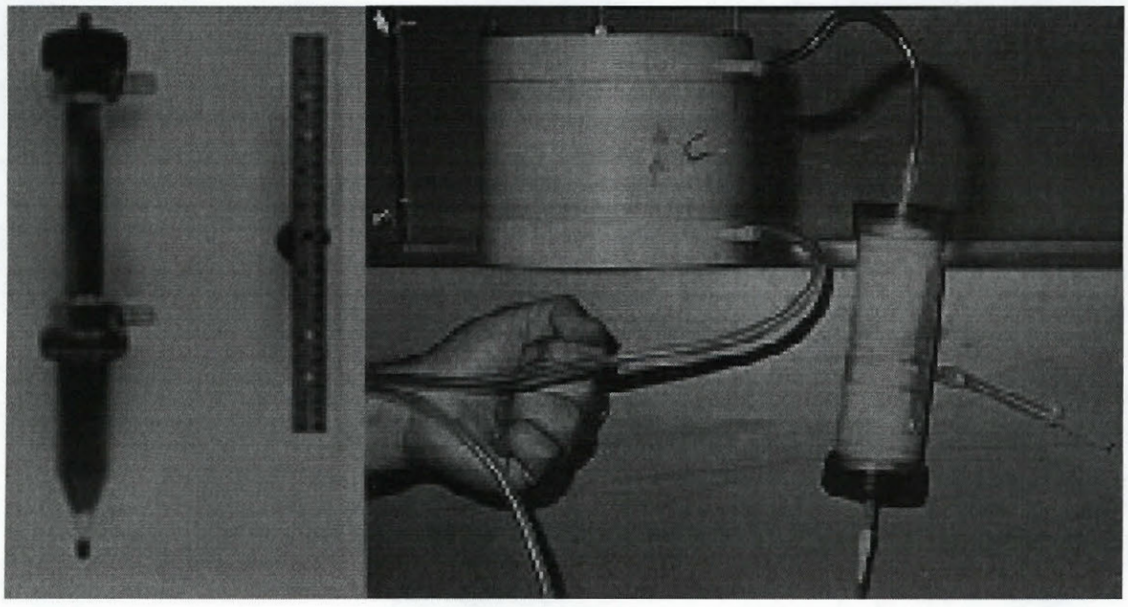
1.3.3.3 There are other approaches like hemofiltration, hemodiafiltration, etc. are available but they are not within the scope of this introduction.

1.3.4 Hemoperfusion

Hemoperfusion is the term applied to the passage of anticoagulated blood directly over adsorbent (sorbent) particles contained in a column device. The sorbent, chemical that adsorbs unwanted metabolites, is used in the extracorporeal circuits. Yatzidis, in 1964, reported the alleviation of uremic sign and efficient removal of creatinine and uric acid by passage of blood over 200 g of activated charcoal contained in a siliconized glass cylinder for 60 min (Yatzid, 1964). Unfortunately, this can not be used in patients because of particulate embolism from charcoal and also platelets removal from the blood.

Chang solved these problems by applying the principle of artificial cells to coat small activated charcoal granules with an ultrathin (200 A^0) synthetic polymer membrane (Chang, 1966,1969,1972). This prevents release of charcoal powder and also prevents adverse effects of charcoal on blood cells (Chang, 1966,1969a).

Since then many modifications have been attempted (Chang, 1966, 1967, 1984,1994,1995; Chang et al., 1974; Courtney et al., 1976, 1978, 1994,1995; Mataha et al., 1995; Asher, 1980). The original method is still the most commonly used one (Chang, 1964, 1994, 1995; Mataha et al., 1995). Since then artificial cells have been used as the basis of a blood detoxifier for hemoperfusion (Chang, 1966, 1967, 1972, 1984a, 1984b, 1991, 994,1995). Artificial cells containing activated charcoal have also been found to maintain terminal renal failure patients alive and alleviate their uremic symptoms (Chang et al., 1971, 1984, 1989; Friedman, 1984; Sparks, 1971). However, this approach does not remove water, electrolytes, and urea. To solve this problem, the blood detoxifier was used in series with the dialysis machine (Chang et al., 1975). With this combination, time of treatment was cut down to 2 hours each session, 3 times a week.



A B

Figure 1.5. A schematic illustration of hemoperfusion device and process developed by Chang. A: Hemoperfusion device; B: Ongoing hemoperfusion process. *Source: Chang, 1976.*

In an effort to eliminate the bulky and expensive dialysis machine, Chang et al. (1977) developed another approach by combining the blood detoxifier with a small ultrafiltrator. This combined hemoperfusion and ultrafiltration system is now the smallest artificial kidney available. The blood detoxifier removes uremic toxins while the ultrafiltrator eliminates water and sodium chloride. However, this approach still requires the removal of urea and electrolytes like potassium and phosphate (Chang et al., 1977, 1984). Potassium and phosphate can be removed by the administration of oral adsorbents (Chang et al., 1977,1984; Friedman, 1980). But the problem for urea removal still remain (Chang, 1977, 1992, 1993, Friedman, 1985, 1995). The requirement for a suitable urea removal system that can be used either alone or in combination with other treatment methods is, therefore, important.

1.3.5 Requirement for Urea Removal

1.3.5.1 General

The major functions of the kidney are to excrete waste products resulting from protein metabolism and to regulate water and electrolyte balance. In patients with chronic renal failure, the capacity of the kidney to fulfil these functions becomes progressively limited. Urea is a major waste product derived from protein metabolism. Because of the diminished glomerular filtration rate, urea tends to accumulate in the body (Mitch, 1983; Guyton, 1995).

The increase in plasma urea and symptoms mimicking chronic nephritis were first found by Prevot and Dumas in 1821 in nephrectomized animals. Subsequently, Bostock in 1826 and Christison in 1829 found increased concentration of blood urea in patients with degradation of kidneys (Schreiner and Maher, 1923). The symptom complex was attributed

to the urea retention and the termed uremia was coined by Piorry in 1840 indicating urine in the blood. Uremia, the clinical state of being poisoned by products of endogenous catabolism during kidney failure and other diseases is characterised by an elevated level of urea and other uremic toxic waste (Shaul, 1986). The increase in blood urea, the "Urea toxic theory", is characterised by a number of clinical and biochemical abnormalities which occur in patients with advanced renal failure (Shaul, 1986).

Clinically a high concentrations of urea have been reported to induce headache, fatigue, nausea, vomiting, glucose intolerance and bleeding tendency (Bergstrom and Furst, 1979). In addition, urea is one of the few substances which exerts in-vitro toxic effects at concentration found in the blood of uremic patients (Bergstrom and Furst, 1979, Massry, 1986). However, it should be stressed that the most severe uremic gastrointestinal cardiovascular, mental, and neurological changes were not noticed in patients dialysed against on a short term basis high urea concentrations (Bergstrom and Furst, 1979). Blood urea level has been used as a marker for adequacy of hemodilaysis treatments. It is likely that many waste uremic metabolites are responsible for problems in uremia.

1.3.5.2 Alternative to Hemodialysis for Urea Removal

Alternatives to hemodialysis for urea removal are still in the experimental stage (Friedman, 1995). These methods can be divided into three categories: (1) adsorbents that bind urea directly, 2) coimmobilized urease and an adsorbent which adsorbs the ammonium ions liberated from urea or coimmoblized urease with multienzyme system and 3) colonization of soil urea utilising of mixed bacterial cells in the intestinal flora.

One of the most widely studied adsorbents for binding urea is oxystarch (Giordano et al., 1972; Friedman, 1980; Martin and Courtney, 1977). Oxystarch is prepared by oxidizing anhydrous starch with periodic acid yielding a polymeric aldehyde derivative. Giordano et al. (1972) pioneered the use of oxystarch as an oral adsorbent for

removing urea. Since then oxystarch has been extensively studied (Friedman et al., 1974, 1975, 1976; Giordano, 1980; Giordano et al., 1975, 1976; Man et al., 1976; Meriwether and Kramer, 1976; Martin and Courtney, 1977; Sparks et al., 1971). However, this polyaldehyde resulted in only a small reduction in systemic urea level when ingested. Furthermore, the ingestion of a large amount of oxystarch produced side effects, such as diarrhea, nausea, and intestinal cramps.

The enzymatic conversion of urea into ammonia, which is then removed by an adsorbent, constitutes another method of removing urea. Chang (1966) demonstrated the effectiveness of microencapsulated urease in reducing urea levels in animals. The resulting ammonia could be removed by coencapsulated ion exchange resins (Chang, 1966,1972; Sparks et al., 1969). Gordon et al., (1971) utilized this method in the REDY system for dialysate regeneration. Oral ingestion of microencapsulated urease with ammonium adsorbent lowered systemic urea levels in rats (Chang and Loa, 1970). Gardner's group developed this approach for use in patients. They used a combination of microencapsulted urease immobilized on silica and an ion exchange resin, zirconium phosphate. This approach showed a reduction in urea in patients from 75 to 60 mg/dl (Gardner 1984; Kjellestrand et al., 1981). However, the oral dose of zirconium phosphate required for urea removal is too large to be readily tolerated by patients.

Another approach is the use of intestinal colonisation of mixed soil bacterial cells by Setala (Setala 1970, Setala et al., 1971, 1973). This is found not sufficiently effective (Gardner 1984; Freidman, 1984, 1989).

The requirement for an inexpensive alternative of existing urea removal system is, therefore, important. It is very urgent particularly for the people residing in the developing countries where the majority of population simply cannot afford the standard dialysis treatment (Freidman, 1995). My Ph.D. thesis is, therefore, conducted with the aim of uncovering a new method for urea removal.

1.4 AMMONIA REMOVAL IN LIVER FAILURE

1.4.1 Introduction

The liver is a vitally important organ for the normal functioning of the body. The liver is organised in lobules, within which blood flows past hepatic cells via sinusoids from branches of the portal vein to the ecentral vein of the each lobule. The endothelium of the sinusoid has large fenestration, and plasma is in close contact with the liver cells, as the hepatic blood flow is 1,500 ml/min (Ganong, 1994). The hepatic circulation lies between the venous drainage of the intestinal tract and the systematic circulation. Therefore almost all substances absorbed from the intestinal tract pass through the liver via the portal blood before reaching the systemic circulation (Jensen, 1976; Ganong, 1994). The substances in the blood coming from the gastrointestinal (GI) tract are altered chemically by the liver into forms that are suitable for direct use or for further processing by other specialised tissues or organs. For example, ammonia in the blood from the GI is converted to urea by the Krebs cycle enzymes in the liver (Krebs et al., 1978; Wolpert, Philips, and Summerskill, 1971). The kidneys are then able to excrete ammonia as urea with the urine.

Liver is responsible for the synthesis of numerous proteins and enzymes needed to maintain homeostasis (Uchino, 1994). Other major functions include the formation of bile; carbohydrate storage, control of lipid, amino acids, peptide and carbohydrate metabolism; reduction, conjugation of adrenal and gonadal steroid hormones; detoxification; synthesis of plasma proteins; and many other important functions.

The complications of hepatic failure relate the inability of the liver to continue its vital synthetic, metabolic, and detoxification function. Synthetic failure results in a decrease in circulating albumin and component proteins of coagulation cascade. The latter results in bleeding diathesis, the former in decreased in plasma oncotic pressure, and tissue edema. Metabolic failure results in a build up of bilirubin, a product of hemoglobin degradation. There is also an increase in ammonia and other toxins. One form of liver disease is chronic,

and results from progressive, persistent damage as seen in viral infection, alcoholism, and autoimmune attack. Alcoholic liver disease is the most common etiology in N. America, whereas viral hepatitis is most common in Asia (Uchino,1994). Progressive hepatocelluler injury leading to cycles of necrosis, scarring and disordered regeneration give the histopathological cirrhosis. Clinically, the condition is characterised by a build-up of fluid in the peritoneal cavity, or ascites, from prolonged portal venous hypertension and hyperproteionemia. Also, decreased ability to clear bilirubin from the blood stream results in jaundice. With progression of the disease, the victims are prone to, among other complications, massive upper gastrointestinal haemorrhage from portal systemic anastomoses and the development of encephalopathy and coma from the build up of toxic metabolites (Uchino,1994). Beside, disturbed mental functions including coma, intellectual deterioration and slow and slurred speech characterises liver failure (Sherlock, 1989; Zakim and Boyer, 1990). While some of the complications of chronic liver disease can follow a rapid course, the disease is generally insidious evolving over months to years.

There are several degrees of liver failure. Important criteria of its characterisation is the presence or absence of hepatic encephalopathy (Trey et al., 1970; Bernuau et al., 1986). Hepatic encephalopathy is a disorder of neuromuscular functions and consciousness occurring in patients with liver disease as described above (Zieve, 1982). This disorder may be graded in order of increasing severity from I to IV (Zieve, 1982; Duffy, 1982). The exact cause of hepatic encephalopathy remains obscure (Herlong and Diehl, 1983; Trey et al., 1970; Bernuau et al., 1986).

There are at least 14 categories of metabolic disturbances which occur during liver failure (Zeive, 1975). The complexity of disturbances have not allowed the identification of the precise metabolic event or events which cause hepatic encephalopathy. However, since liver failure complicated by encephalopathy has such a high rate of mortality, about 80 %, the question of what causes encephalopathy has received a lot of attention (Zeive,1975; Guyton, 1995; Hugues and Williams, 1981).

The concentration of all the amino acids, bilirubin, middle molecule substances (mol. wt. 400-1500 Daltons), neurotransmitters and other substances are elevated in the blood plasma during liver failure (Zeive, 1975; Hugues and Williams, 1981; Guyton, 1995).

The increase in the concentration of ammonia, mercaptans, and fatty acids have been suggested as a possible cause of hepatic encephalopathy (Record et al., 1975). In end stage liver disease, the only treatment is supportive measures. Eventually, the last resort would be either to go for liver transplantation, which is a definitive therapy for liver disease, or to use artificial liver support (ALS) systems (Dodson et al., 1994).
In general, hepatic support can be achieved by two fundamental approaches :

1. Nonbiological approach
2. Biological approach

1.4.2 Non Biological Artificial Liver Support Systems

Non-biological liver support involves methods such as hemoperfusion, hemofilteration, hemodialysis, sorption, exchange transfusion, plasma exchange , plasma fractionation, and pharmacological protection using drugs such as prostaglandins (Chang, 1884; Malchesky, 1994). The objective of the artificial liver support (ALS) system is to restore the plasma composition. The philosophy underlying the ALS system, therefore, is that certain toxins (e.g. ammonia, bilirubin, methionine, etc.) ought to be removed from, and certain critical chemicals (e.g. blood clotting factors etc.) ought to be provided to the liver failure patient (Knell, 1981). If this is done, it is hoped that the patient's life can be prolonged until his liver regenerates or he receives a suitable liver donor.

The use of exchange transfusion, to remove toxic or waste metabolites was reported as early as 1958 for the hepatorenal syndrome and in 1958 for hepatic coma (Snapper et al., 1958; Lee et al., 1958). By 1972 results of 185 patients treated using exchange transfusion from 38 centres across the world, shows survival of only 31 %, which was not significantly different than the patients receiving conservative treatment (Kuster, 1976). The clinical use of total body fluid wash out, which was first reported in 1972 (Klebonoff, 1972), and cross circulation has also been summarised even less effective in decreasing the mortality

(Mohsini et al., 1980). In 1972, Chang treated grade IV hepatic coma patients using a cellulose nitrate coated charcoal hemoperfusion systems (Chang, 1972, 1973). This resulted in recovery of consciousness from the IV grade hepatic coma. This was supported by other groups(e.g. Williams et al.,1975). However, it was not effective in long term survival in grade IV hepatic coma (Chang and Migchelsen, 1973; Williams et. al, 1975).

Charcoal based hemoperfusion system is effective clinically in removing middle molecules (M.W. 300-1500 Dalton), mercaptans, gamma-butyric acids, phenols, aromatic amino acids, inhibitors of Na and K ATPase activity and a large spectrum of potentially toxic substances (Chang, 1986; Chang and Migchelsen, 1973; Williams et al., 1975). Ash used charcoal in a membrane device and found that it is effective in significantly improving neurological status of the patients in fulminant failure, with approximately 50 % of these patients achieving the goal of successful support until regeneration of hepatic functions or transplantation (Ash, 1994). It is advantageous in its simplicity and lack of adverse effects, particularly when used after microencapsulation. Its constraint is that it only carries out the detoxifying function of the liver. Also it can not remove sufficient ammonia. In general, the major disadvantage of non-biological systems is that they fail to support the essential hepatic functions of biosynthesis or biotransformation and so far the overall survival rate of grade IV hepatic coma patients with fulminant hepatitis has not exceeded more than 30 % with the treatment using clinically available artificial liver supports, such as haemodialysis, hemoperfusion, hemofiltration, and plasmapheresis (Ash, 1994; Dodson et al., 1994).

1.4.3 Biological Artificial Liver Support Systems

The biological approach of artificial liver support includes orthotropic and heterotropic liver transplantation, bioartificial liver using cultured hepatocytes, microencapsulated hepatocytes and cross circulations.

A definite therapy for liver disease is orthotropic liver transplantation (Uchino and

Matsushita, 1994). Liver transplantation was carried out clinically in 1963 and by the end of 1975 about 220 patents had received liver transplants (Starzl, 1963; Kuster, 1976). Since its earliest trials in 1960's, this procedure has seen major advances in success rate, particularly in the last 15 years with the development of improved surgical techniques, and the immunosuppressive agents (Dodson et al., 1994). Currently, the 5 year survival from liver transplantation for all causes of failure rests at approximately 70-75 % (Dodson et al., 1994). Although this is a truly remarkable achievement, as with other forms of therapy, the practice of liver transplantation still has its disadvantages which prevents it from routine clinical practice.

The major problems associated with this form of liver failure management include donor scarcity, expense, technical difficulty in surgery, complex care, immunorejection, hemodynamic instability of the patients, particularly in fulminant failure. Donor scarcity, in particular, is a major concern, because it has been attributed to so many deaths while the patients become critically ill in the process of waiting. It is estimated that as many as 3,000 liver patients in USA, and 2,300 in Europe are waiting in year 1993 alone for suitable donor and also it is reported that the scarcity of the donors is getting more severe and 10-30 % patients are reported to die before grafts become available (Kasai,1994; Malchesky, 1994). But there is proposal of theoretical advantages of using hepatocyte transplantation as a potential mean of liver function replacement (Demetriou, 1994).

1.4.3.1 Hybrid as Artificial Liver Support

Effort to develop a biohybrid artificial liver that could detoxify blood and provide necessary synthetic functions begins in the late 1950's. Nose' used canine liver preparations in various forms such as slices, tissue homogenate and freeze-dried granules, placed in the dialysate compartment of dialysis machine. He was able to document the ability of this bioreactor to maintain plasma glucose concentration and lower otherwise toxic levels of ammonia in the patients (Nose et al.,1972; Takahasi et al., 1991). Some of the most

promising results using bioartificial liver devices have been obtained by Demetriou's group (Demetriou and Bollinger. 1986; Neuzil et al., 1993; Rozga et al., 1993,1994). These systems can marginally lower the metabolic toxic level and produce the liver synthesising factors. The lack of oxygen, nutrients and the immunorejction are the most often reported causes of the their limited success (Harland and Bollinger, 1994). There are several problems common to these artificial liver models :

1. how to maintain the viability of the hepatocytes for long period of time,
2. how to store cells for a long period of time prior to use,
3. how to control immunounological hazards and biocompatibility,
4. how to increase the density of the cultured cells, as 30 % of the total liver biomass (25-75 gm of the hepatocytes) is needed to sustain life (Kasai et al., 1994), and
5. how to provide adequate perfusion to the implanted cells ?

1.4.3.2 Bioencapsulated Hepatocytes

Chang first proposed the use of microencapsulated hepatocytes for immunoisolation (Chang, 1965). In 1965, he wrote : "the enclosed materials might be protected from destruction and from participation in immunological processes, while the enclosing membrane would be permeable to small molecules of specific cellular product, which could then enter the general extracellular components of the recipient. For instance, the encapsulated endocrine cells might survive and maintain an effective supply of hormones....the situation could then be comparable to that of a graft placed in an immunologically favourable site" (Chang, 1965). In microencapsulation systems hepatocyte cells are placed into a semipermeable microcapsule. The encapsulated hepatocytes then could be used in number of ways such as transplanted into the peritoneal cavity (Bruni and Chang, 1989,1991,1995; Dixit, 1994; Wong and Chang, 1988a, 1988b), in hallow fibre bioreactor (Miura et al., 1987; Yaungi et al., 1989), or be injected into the

portal system and reside in the sinusoid of the liver (Burner, 1981; Grosmman et al., 1993). Microencapsulated hepatocytes when implanted into experimental animals results in immunoisolation and the prolonged survival and function of hepatocyte cells (Wong and Chang, 1986,1988a,1988b; Bruni and Chang, 1995; Dixit, 1994).

The other benefits of microencapsulated hepatocytes includes easier handling, storage, and increased biocompatiability (Chang, 1971,1992,1994; Lim and Sun, 1980; Wong and Chang, 1986,1988,1991; Langer, 1993; Dixit, 1994, Bruni and Chang,1989, 1991, 1995). Wong and Chang reported the increased survival time after the implantation of 15 million microencapsulated hepatocytes within the peritoneal cavity of liver failure rat (Wong and Chang, 1986). Fifty million microencapsulated hepatocytes, were reported to increase the survival rate in liver failure rats when implanted intraperitoneally (Sun et al., 1986). In 1995, Bruni and Chang reported the use of microencapsulated hepatocytes for the treatment of bilirubinemia in Gunn rats (Bruni and Chang, 1989,1991,1995).

1.4.4 Requirement for Ammonia Removal

1.4.4.1 Ammonia : One of the Toxic Metabolites

Ammonia is formed in the tissues and colon, and absorbed into portal venous blood (Ganong, 1994). Ammonia, in the hepatic portal blood from the GI tract, is usually metabolised by the liver, where it is processed into urea via the urea cycle (Zakim and Boyer, 1990; Guyton, 1995). Hence, the blood leaving the liver is virtually ammonia free. The linkage of liver disease to the systemic ammonia level is strong (Zeiv et al.,1974; Zakim and Boyer, 1990). For a long time the "ammonia intoxication theory" had dominated in the field of liver failure research. However, the mechanism by which ammonia causes deleterious effects is still unknown (Minana et al., 1995). Also, the exact rate of ammonia toxicity in liver failure has not been established. Indeed, like ammonia, other "toxic"

substances have also been proposed but their toxic effects are also still under debate (Hayes, 1994). When the liver is diseased, ammonia is not removed from the blood, and hyperammonemia is the result. It has been proposed that this can cause abnormal neurological symptoms (hepatic encephalopathy). A number of basic cellular mechanism have been suggested as possible causes of ammonia toxicity. These include:

1) interference with brain energy metabolism (Walker and Schenker, 1970),
2) accumulation of inhibitory neurotransmitters, gamaaminobutyric acid (Goetchus and Webester, 1965),
3) decrease in the neuritransmitter acetylcholine (Braganca,1953),
4) direct inhibitory effect on neural membrane (Knegt et al., 1995), and
5) other mechanisms.

In liver failure, ammonia acts synergistically with mercaptans and fatty acids. When they are present together, a much smaller amount is required to induce coma. The increase in the concentrations of ammonia, mercaptans, and fatty acids have been suggested as a likely cause of encephalopathy which commonly occurs during liver failure (Zeive, 1975; Zeive, 1981; Raaby, 1989). The deleterious effect of ammonia is not limited to its ability to precipitate coma but it also increases the permeability of the blood brain barrier and involved in cerebral edema development (Zaki et al., 1988). The other damaging effect of ammonia is that it inhibits the regeneration of the liver (Zieve et al., 1975; Zakim and Boyer, 1990). The elevated ammonia level in a neonatal period is commonly associated with significant central nervous system damage (Minana et al.,1995).

1.4.4.2 Current Approaches for Ammonia Removal

Attempts to remove ammonia, by hemodialysis, in patients suffering from ammonia intoxication was started as early as 1958 (Kieley et al., 1958). Polyhalides, such as oxidized

starch and cellulose, are very potent urea and ammonia binders. Ion exchange resins, especially cations, have been tested for the removal of ammonia (Schechter, 1958; Nealon and Chang, 1964; Chang, 1966; Ritchi, 1962; Juggi, 1972; Friedman, 1974,1980,1984; Cipoletti et al., 1980). Animal studies have shown that ion exchange resins are effective in removing ammonia from the plasma of the animals (Schechter, 1958; Nealon and Ching, 1964; Ritchi, 1962; Juggi, 1972). However, the associated properties of ion exchange resins to bind other essential ions, such as $Ca\ 2^{+}$, $Mg\ 2^{+}$ and K^{+}, platelets, leukocytes etc. from the perfused blood has put them aside from the routine clinical application of direct blood perfusion (Roesenbaun et al., 1970; Chang, 1984). However, they can be used together with plasmapheresis where blood cells are not present and therefore not damaged.

The indirect approach includes oral administration of antibiotics such as neomycin, kanamycin, etc. to reduce ammonia production by bacteria in the large intestine (Bower, 1987), combination of enzyme and hemoperfusion (Chang, 1989,1992,1993), microencapsulated multienzyme system (Chang et al.,1979, 1986, Chang, 1992, 1993; Cousineau and Chang, 1982; Gu and Chang, 1986; Yu and Chang, 1982), etc..

None of these are sufficiently effective. A more efficient method of ammonia removal is, therefore, needed.

1.5 BACTERIA FOR UREA AND AMMONIA REMOVAL

The main component of the global nitrogen cycle in nature involves an eight electron shift between nitrate, the most oxidised form, and ammonia, the most reduced form and in the form in which nitrogen is assimilated in the microorganisms (Madarak and Kunst, 1988). Using this property, removal of nitrogenous compounds such as urea and ammonia by urea and ammonia using bacteria for waste water treatment is potentially possible (Mara and Pearson, 1986; Mudarak and Kunst,1988; Ladish et al., 1992). There are several hundred species of bacteria and other microorganisms that are known to assimilate urea and ammonia for their daily nutritional nitrogen requirements (Bergey's,

1937,1978,1994).

Setala (1972) screened 729 strains of soil bacteria that specifically biodegrade in-vitro high quantity of non-proteinious nitrogen (NPN) that accumulates in tissues of renal failure patients. He narrowed and concentrated on the 5 strains of Sereeatia species that had a high capacity of urea decomposition (Setala, 1973; Setala et al. 1973). When given, after lyophilization, to the uremic patients for alleviation of uremic symptoms, these bacteria were shown to be able to decrease systemic urea, creatinine and other NPN compounds (Setala et al. 1973). The efficiency of these mixed bacterial system was, however, too low to allow for routine clinical applications.

Immobilized microbial cells have also been used for the removal of nitrogenous waste. Sumino et al. (1992) showed no effect of immobilization materials, urethane prepolymer, on the activity of nitrifying bacteria and removal of ammonium nitrogen from waste water and the life of the bacterial pellets were reported as long as 120 days (Suminoi et al., 1992). A column bioreactor, along with a sensitive ammonia monitoring device, for the removal of ammonia from waste water is also reported (Hikuma et al., 1992). Other groups (Xing et al., 1995) have designed a continuous flow fluidized-bed bioreactor system for the simultaneous removal of carbonaceous and nitrogenous substances in waste water. The use of bacteria, *Erwinia herbicola,* for the conversion of ammonia along with phenol and pyruvate, to tyrosine is also reported (Lloyd-George and Chang, 1995). The ammonia conversion rate for this bacteria was reported, however, very low (Lloyd-George,1993; Lloyd-George and Chang,1993, 1995).

Genetic engineering, a technique of changing genes using an in-vitro process, has been established to enhance many specific characteristics in many types of cells including bacteria (Ladish et al., 1992; Robert et al.,1995). This technology is particularly useful when one wishes to introduce a gene or its part from a higher organism into bacteria or from one bacteria to another, but under the control of a stronger promoter, part of the gene coming from the third organism usually for enhanced expression of the introduced gene. Using this technology several manipulations have been done in many bacterial species for the enzyme urease which converts urea into ammonia which then enters into the bacteria's metabolic pool for the biosynthesis of proteins etc. Many reviews are available (Bakheet and Syrett,

1977; Kim and Spizizen, 1985; Mobley et al., 1989;1995). The genes responsible for urea metabolism have been cloned from bacteria *Bacillus pasteurii* (Kim and Sizizen, 1985), *Proteous miranilis* (Jones and Mobley, 1988; Waltz et al., 1988) and *Providencia stuartii* (Mulrooney et al., 1986; 1988). One of the best-studied bacterial urea using enzyme is from *Klebsiella aerogens* (Mulrooney et al., 1989). The gene from *Klebsiella aerogens* is been cloned into bacteria *Escherichia coli,* a bacteria about which we know the most today amongst all the bacterial species (Mathew and Haussinger, 1987; Mulrooney et. al, 1989). This has resulted in several thousand fold enhancement in urea and ammonia using capacity of *Escherichia coli* (Mathew and Hausinger, 1987; Mulrooney et al., 1988; 1989).

In this thesis, research has been designed to combine the principle of artificial cells with the urea utilising efficiency of genetically engineered bacteria *Escherichia coli* DH 5 cells which have genes from *Klebsiella aerogens.* This research, if successful, may uncover a new way to use unique properties of genetically engineered cells.

1.6 AIMS AND OBJECTIVES

The concise statements of the studies can be summarised as follows:

1) To develop a novel approach involving the use of artificial cells containing genetically engineered bacterial cells for biomedical applications.

(2) To optimize the microencapsulation process parameters for genetically engineered bacterial cells immobilization by microencapsulation.

(3) To develop a new approach involving the use of artificial cells, containing genetically engineered bacterial cells in order to remove urea and ammonia for potential bioengineering and biotechnology applications.

(4) To develop a new method of urea and ammonia removal in kidney failure, liver failure and other diseases using the proposed new approach of using artificial cells containing genetically engineered bacterial cells.

(5) To evaluate the feasibility of this new approach for both in-vitro and in-vivo urea and ammonia removal. This will then serve as an example for the use of artificial cells containing other types of genetically engineered bacterial cells for future applications.

1.7 REFERENCES

Agishi, T., Yamashita, N., and Ota, K. (1980). Clinical results of direct charcoal hemoperfusion for endogenous and exogenous intoxication. In Hemoperfusion Part 1, Kidney and Liver Support and Detoxification (Sidemen, S., and Chang, T.M.S., eds.), 255-263, Hemisphere, Washington, D.C.

Aisina, R.B., Kazanskaya, N.F., Lukasheva, E.V., and Berezin, I.V. (1976). Production and properties of microencapsulated a-chymotrypsin. Biokhimiya, 41, 1656-1661. Artif. Organs. 6(4): 178-182.

Ash, S. (1994). Hemodiaadsobtion in treatment of acute hepatic failure. ASAIO journal 40(1): 80-82.

Bakheet, I.A., and Syrett, P.J. (1977). Urea-degrading enzymes. Br. Phycol. J., 12, 137-143.

Bangham, A.D., Hill, M.W., and Miller, and N.G.A. (1974). Preparation and use of liposomes as models of biological membranes. In: Methods in Membrane Biology (Korn, E.D., ed.), 1-68, Plenum Press, New York.

Baricos, W.H., Chambers, R.P., and Cohen, W. (1975). Considerations on the use of cofactor-requiring enzymes in enzyme engineering. Enzyme Technol. Dig., 4(2), 39-53.

Bergy's (1937,1978,1994). In: Bergy's manual of determinative bacteriology (Buchnana, R. E. and Gibbons, coed.), Baltimore, William's and Wilkins Co.

Bernau, J., Rueff, B., and Benhamou, J. (1986). Fulminant and subfulminant liver failure : definition and causes. Seminar in liver diseases 6(2): 97-106.

Blanch, H.W., and Klibanov, A.M. (1988). Enzyme Engineering. Vol.9, The New York Academy of Sciences, New York.

Boag, A.H., and Sefton, M.V. (1987). Microencapsulation of human fibroblasts in a water-insoluble polyacrylate. Biotechnol. Bioeng., 30, 954-962.

Bostock quoted by Quinan, J. R. (1880). Maryland Med. J. Vol.7, 217-224.

Bourget, L., and Chang, T.M.S. (1986). Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. Biochem. Biophys. Acta, 883, 432-438.

Bower, R. H. (1987). Nutritional support in critical care, hepatic failure. Chapter 11:221-231.

Braganca, B.M., Faulkner, P., and Quastel, J. H. (1953). Effects of inhibitors of glutamine synthase on the inhibition of acetylcholine synthesis in brain slices by ammonium ions. Biochem. Biophys. Acta, 10, 83-88.

Briggs, P. J., and Schnermann, B. J. (1994). Overview of renal function: In Premiers on kidney diseases. 1-2 (Grinberg, A., ed.), Academic Press, New York.

Brunner, G. (1982). Advances in the development of immobilized enzymes for future extracarporeal liver support. In : Burnet et al. (eds.). Artificial liver support, Heidelberg spring-Verlag, 230-235.

Blumenkrantz, M.J., Gordon, A., Roberts, M., Lewin, A.J., Pecker, E.A., Moran ,J.K., Coburn, J.W., Maxwell, M.H. (1979). Applications of the Redy sorbent system to hemodialysis and peritoneal dialysis, Artificial Organs, Aug. 3(3):230-236.

Bruni, S., and Chang, T.M.S. (1989). Hepatocytes immobilised by microencapsulation in artificial cells: effects on hyperbilirubinemia in Gunn rats, Biomat., Artif. Cells, & Artif. Org. 17(4):403-411.

Bruni, S., and Chang, T.M.S. (1991). Encapsulated hepatocytes for controlling hyperbilirubinemia in Gunn rats, Int. J. of Artif. Org. 14(4):239-241, 1991.

Bruni, S., and Chang, T.M.S. (1995). Kinetic analysis of UDP-glucuronosyltransferase in bilirubin conjugation by encapsulated hepatocytes for transplantation into Gunn rats, Artificial Organs, 19(5):449-457.

Cai, Z.H., Shi, Z.Q., O'Shea,G .M., and Sun, A.M. (1988). Microencapsulated hepatocytes for bioartificial liver support. Artif. Org., 12, 388-393.

Campbell, J., and Chang, T.M.S. (1975). Enzymatic recycling of coenzymes by a multienzyme system immobilized within semipermeable collodion microcapsules. Biochem. Biophys. Acta, 397, 101-109.

Campbell, J., and Chang, T.M.S. (1976). The recycling of NAD^+ (free and immobilized) within semipermeable aqueous microcapsules containing a multienzyme system. Biochem. Biophys. Res. Commun., 69(2), 562-569.

Cattaneo, M.V. and Chang, T.M.S. (1991).The potential of a microencapsulated urease-zeolite oral sorbent for the removal of urea in uremia., ASAIO Transactions , Apr-Jun; 37(2),80-87

Chang, T.M.S. (1957). Hemoglobin Corpuscles (Report of Research Project for B.Sc., Honors Physiology), McGill University, Montreal, Canada.

Chang, T.M.S. (1964). Semipermeable microcapsules. Science, 146, 524-525.

Chang, T.M.S. (1965). Semipermeable Aqueous Microcapsules (Ph.D. Thesis), McGill University, Montreal, Canada.

Chang, T.M.S. (1966a). Semipermeable aqueous microcapsules (artificial cells): With emphasis on experiments in an extracorporeal shunt system. Trans. Am. Soc.Artif. Intern. Org., 12, 13-19.

Chang, T.M.S., MacIntosh, F.C., and Mason, S.G. (1966b). Semipermeable aqueous microcapsules: I. Preparation and properties. Can. J. Physiol. Pharmacol., 44, 115-128.

Chang, T.M.S., Johnson, L.J., and Ransome, O. (1967). Semipermeable aqueous microcapsules: IV. Nonthrombogenic microcapsules with heparin-complexed membranes. Can. J. Physiol. Pharmacol., 45, 705-715.

Chang, T.M.S., and Poznansky,M.J. (1968). Semipermeable microcapsules containing catalase for enzyme replacement in acatalasaemic mice. Nature (London), 218, 243-245.

Chang, T.M.S. (1969a). Removal of endogenous and exogenous toxins by a microencapsulated adsorbent. Can. J. Physiol. Pharmacol., 47(12), 1043-1045.

Chang, T.M.S. (1969b). Lipid-coated spherical ultrathin membranes of polymer or cross-linked protein as possible cell membrane models. Federation Proceedings, 28(2), 461.

Chang, T.M.S. (1969c). Clinical potential of enzyme technology. Science Tools, 16(3), 33-39.

Chang, T.M.S. (1969d). Asparaginase-loaded semipermeable microcapsules for mouse lymphomas. Proc. Can. Fed. Biol. Sci., 12, 62.

Chang, T.M.S., and Loa, S.K. (1970a). Urea removal by urease and ammonia absorbents in the intestine. The Physiologist, 13(3), 70-72.

Chang, T.M.S., and Malave, N. (1970b). The development and first clinical use of semipermeable microcapsules (artificial cells) as a compact artificial kidney. Trans. Am. Soc. Artif. Intern. Org., 16, 141-148.

Chang, T.M.S. (1971a). The in-vivo effects of semipermeable microcapsules containing L-asparaginase on 6C3HED lymphosarcoma. Nature, 229, 117-118.

Chang, T.M.S., Conda, A., Dirks, J.H., and Malave, N. (1971b). Clinical evaluation of chronic, intermittent, and short term hemoperfusions in patients with chronic renal failure using semipermeable microcapsules (artificial cells) formed from membrane-coated activated charcoal. Trans. Am. Soc. Artif. Intern. Org., 17, 246-252.

Chang, T.M.S. (1972a). Artificial Cells. Thomas, Springfield, Illinois.

Chang, T.M.S. (1972b). Hemoperfusion over microencapsulated adsorbent in a patient with hepatic coma. Lancet II, 1371-1372.

Chang, T.M.S., Conda, A., Dirks, J.H., Coffey, J.F., and Lee-Burns, T. (1972c). ACAC microcapsule artificial kidney for the long term and short term management of eleven patients with chronic renal failure. Trans. Am. Soc. Artif. Intern. Org., 18, 465-472.

Chang, T.M.S. (1972 d). Hemoperfusion over microencapsulated adsorbent in a patient with hepatic coma. Lancet II, 1371-1372.

Chang, T.M.S., and Migchelsen, M. (1973a). Characterization of possible "toxic" metabolites in uremia and hepatic coma based on the clearance spectrum for larger molecules by the ACAC microcapsule artificial kidney. Trans. Am. Soc. Artif. Intern. Org., 19, 314-319.

Chang, T.M.S. (1973b). L-Asparaginase immobilized within semipermeable microcapsules: In-vitro and in-vivo stability. Enzyme, 14(2), 95-104.

Chang, T.M.S., Chirito,E., Barre, P., Cole,C., and Hewish, M. (1975). Clinical performance characteristics of a new combined system for simultaneous hemoperfusion- hemodialysis-ultrafiltration in series. Trans. Am. Soc. Artif. Intern. Org., 21, 502-508.

Chang, T.M.S. (1976a). Hemoperfusion alone and in series with ultrafiltration or dialysis for uremia, poisoning and liver failure. Kid. Int., 10, S305-S311.

Chang, T.M.S. (1976b). Microcapsules artificial kidney: Including updated preparative procedures and properties. Kid. Int., 10, S218-S224.

Chang, T.M.S. (1976c). Biodegradable semipermeable microcapsules containing enzymes, hormones, vaccines and biologicals. J. Bioeng., 1, 25-32.

Chang, T.M.S. (1977). Biomedical Applications of Immobilized Enzymes and Proteins. Vols.1-2, Plenum Press, New York.

Chang, T.M.S., Chirito, E., Barre, P., Cole, C., Lister, C., and Resurreccion, E. (1977). Clinical evaluation of the clearance profiles of a portable, compact, dialysate- free system incorporating microencapsulated charcoal hemoperfusion for blood purification with ultrafiltration for fluid removal. J. Dialysis and Transplant., 8,20-26.

Chang, T.M.S. (1978). Artificial Kidney, Artificial Liver, and Artificial Cells. Plenum Press, New York. 301-304.

Chang, T.M.S., and Malouf, C. (1978). Artificial cells microencapsulated multienzyme system for converting urea and ammonia to amino acid using a-ketoglutarate and glucose as substrate. Trans. Am. Soc. Artif. Intern. Org., 24, 18-20.

Chang, T.M.S., and Malouf, C. (1979a). Effects of glucose dehydrogenase converting urea and ammonia into amino acid using artificial cells. Artif. Org., 3(1), 38-41.

Chang, T.M.S., Malouf, C., and Resurreccion, E. (1979b). Artificial cells containing multienzyme systems for the sequential conversion of urea into ammonia, glutamate, then alanine. Artif. Org., 3, S284-S287.

Chang, T.M.S. (1980). New approaches using immobilized enzymes for the removal of urea and ammonia. In Enzyme Engineering (Weetall, H.H., and Royer, G.P., Eds.), Vol.5, 225-229, Plenum Press, New York and London.

Chang, T.M.S. (1981). Hemoperfusion, exchange transfusion, cross circulation, liver perfusion, hormones and immobilized enzymes. In Artificial Liver Support (Brunner, G., and Schmidt, F.W., Eds.),126-133, Springer-Verlag, Berlin.

Chang, T.M.S. (1982). Earlier hemoperfusion in fulminant hepatic failure, Lancet II, 1039.

Chang, T.M.S. (1983a). Artificial liver support : Requirement and approaches. Int. J. of Artif. Org., 6(4), 178-182.

Chang, T.M.S., and Barre, P. (1983b). Effect of deferrioxamine on removal of Al and Fe by coated charcoal hemoperfusion and hemodialysis. The Lancet, Nov., 1051-1053.

Chang, T.M.S. (1984a). Liver support systems. In : "Life support system in intensive care", Bartelett et al., ed., Year Book Med. Publ., Chicago. 461-485.

Chang, T.M.S. (1984b). Liver support systems. In Life Support Systems in Intensive Care (Bartlett, R.H., Whitehouse, W.M. Jr., Turcotte, J.G., and Harper, M.L., eds.), Year Book Medical Publishers, INC., Chicago.

Chang, T.M.S. (1984c). Artificial cells in medicine and biotechnology. Appl. Biochem. Biotechnol., 10, 5-24.

Chang, T.M.S. (1985a). Artificial cells containing multienzyme systems. In Methods in Enzymology (Widder, K.J., and Green, R., eds.), Vol.112, 195-203, Academic Press, New York.

Chang, T.M.S. (1985b). Biotechnology of artificial cells including application to artificial organs. In Comprehensive Biotechnology (Moo-Young, M., ed.), Vol.4, 53-72, Pergamon Press, New York.

Chang, T.M.S. (1986). Experimental artificial liver support with emphasis on fulminant hepatic failure : Concept and review. Seminars in liver diseases. 6(2), 149-158.

Chang, T.M.S. (1987). Recycling of NAD(P) by multienzyme systems immobilized by microencapsulation in artificial cells. In: Methods in Enzymology (Mosbach, K., ed.), Vol.136, 67-82, Academic Press, New York.

Chang, T.M.S. (1988). Medical applications of immobilized proteins, enzymes, and cells. In Methods in Enzymology (Mosbach, K., ed.), Vol.137, 444-457, Academic Press, New York.

Chang, T.M.S. (1990). " Hemoperfusion, sorbent and immobilized biorectants" Chang, T.M.S. and Odaka, M, eds.). Special symposia volume, Int. J. Biomat. Artif. Cells and Artif. Org. 18, 455-568.

Chang, T.M.S. (1991). "Artificial cells" in encyclopedia of human biology (Renalo Dulbecco ed.), Vol. 1, 377-383, Academic press Inc., San Diego, CA.

Chang, T.M.S. (1992a). Artificial liver support based on artificial cells with emphasis on encapsulated hepatocytes. J. Artif. Org., 16, 71-74.

Chang, T.M.S. (1992b). living cells and microorganism immobilized by microencapsulation inside artificial cells. In: "Fundamentals of animal cell immobilization (Goosen, M.F., ed.), CRC Press.

Chang, T.M.S. (1992c). "Artificial cells 35 years". J. Artif. Org., 16, 8-12.

Chang, T.M.S. (1992d). Artificial liver support based on artificial cells with emphasis on encapsulated hepatocytes. Artificial Organs, 16 (1),71-74.

Chang, T.M.S. (1992e). Artificial cells in immobilization biotechnology. Biomat. Artif. Cells & Immo. Biotech., 20(5),1121-1143.

Chang, T.M.S. (1993a). "Adequacy of hemoperfusion in uremia" in Adequacy of dialysis (Bonomini, V. and Stefoni, eds.).

Chang, T.M.S. (1993b). Adequacy of hemoperfusion for blood purification in uremia. Contributions to Nephrology, 103,10-25.

Chang, T.M.S. (1994). Artificial cells including blood substitute and bioencapsulation, J. Artif. Cells Blood Subs. and Imm. Biotech., 22, vii-xiv.

Chang, T.M.S. (1995). Artificial cells with emphasis on bioencapsulation in biotechnology In: Biotechnology Annual Rev., Vol. 1, 267-295.

Chibata, I. (1978). Immobilized Enzymes, Research and Development. John Wiley & Sons, New York.

Chibata, I., Fukui, S., and Wingard, L.B.Jr. (1982). Enzyme Engineering. Vol.6, Plenum Press, New York.

Chibata, I., and Wingard, L.B.Jr. (1983). Applied Biochemistry and Bioengineering. Vol.4 (Immobilized Microbial Cells), Academic Press, New York.

Christison, R.(1829). Observations on the variety of dropsy which depends on diseased kidneys. Edenburg Med. Surg. J., 32, 262-292.

Cima, L.G , Ingber, D. E. Vacanti, J. P., and Langer R.(1991). Hepatocytes culture on biodegradable polyesteric substitutes. Biotechnology and Bioengineering, 38,143-151.

Cipoletti J. J., Kunin, R. and Francis, M. (1980). Resin technology in medicine. In: Sorbents and their clinical application (Giordano, ed.) academic press, New York.

Cousineau, K., and Chang, T.M.S. (1977). Formation of amino acid from urea and ammonia by sequential enzyme reaction using a microencapsulated multi-enzyme system. Biochem. Biophys. Res. Commun., 79(1), 24-31.

Courtney, J.M, Fairweather, I.A., Gilchrist, T., Hood, R.G., Park, G.B., and Smith, E.M. (1976). The design of a polymer for activated carbon coating. In : Kenedi, R.M. et al. (ed.). Artificial Organs. Baltimore, Univ. Park Press.

Courtney, J.M., Falkenhagen, D. Holtz, M., Schmidt, R., and Klinkmann, H.(1978). Polymer coatings for hemoperfusion over activated carbon. Artificial Organs, 2(3),314-317.

Courtney, J.M. (1993). Artificial organs and biomaterials. J. of Med. Engg. and Tech. 17(5), 188-190.

Courtney, J.M, Sundaram, S., and Matata, B.M., and Gaylor J.D. (1994). Biomaterials in cardiopulmonary bypass. Perfusion, 9 (1),3-10.

Courtney, J.M., Lamba, N.M.K., Gaylor, J.D.S., Ryan, C.J., and Lowe, G.D.O. (1995). Blood- contacting biomaterials - bioengineering viewpoints. Artificial Organs, 9(8), 852-856.

Coromili, V. and Chang, T.M.S. (1993). Polydisperse dextran as a diffusing test solute to study the membrane permeability of alginate polylysine microcapsules, Biomaterials, Artificial Cells, & Immobilization Biotechnology, 21(3), 427-444

Damon, C. (1981). Tissue Microencapsulation. Needham Heights, MA.

Demetriou, A. A. (1994). Hepatocyte transplantation. Scientific American, Dec.,58-67.

Demetriou, A. et al. (1986). Replacement of liver function in rats by transplantation of microcarriers-attached hepatocytes. Science, 23,1190-1192.

Desnick, R.J., Fiddler, M.B., Thorpe, S.R., and Steger, L.D. (1977). Enzyme entrapment in erythrocytes and liposomes for the treatment of lysosomal storage diseases. In Biomedical Applications of Immobilized Enzymes and Proteins (Chang, T.M.S. ed.), Vol.1, 227-244, Plenum Press, New York.

Dinelli, D. (1972). Fiber-entrapped enzymes. Process Biochem., 7(7), 9-12.

Dinelli, D., Marconi, W., and Morisi, F. (1976). Fiber- entrapped enzymes. In Methods in Enzymology (Mosbach, K., ed.), Vol.44, 227-243, Academic Press, New York.

Dixit, V. (1994). Development of artificial liver using isolated hepatocyte. Artificial Organs, 39, 371-389.

Dodson, S. Dehra, K., and Iwastuki, S. (1994). Liver transplantation for fulminant hepatic failure, ASAIO journal, 40(1),74-77.

Drukker, W., Parsons, F.M., and Maher, J.F. (1983). Replacement of Renal Function by Dialysis. Martinus Nijhoff Publishers, Boston.

Fischer, J.E. (1975). On the occurrence of false neurochemical transmitters. In Artificial Liver Support (Williams, R., and Murray-Lyon, I.M., eds.), 31-50, Pitman Medical, London.

Fischer, J.E., Funovics, J.M., Aguirre, A., James, J.H., Keane, J.M., Wesdorp, R.I.C., and Toshimura, N. (1975). The role of plasma amino acids in hepatic encephalopathy. Surgery, 78(3), 276-290.

Fischer, J.E., Rosen, H.M., Ebeid, A.M., James, J.H., Keane, J.M., and Soeters, P.B. (1976). The effect of normalization of plasma amino acids on hepatic encephalopathy in man. Surgery, 80(1), 77-91.

Fischer, J.E., Yoshimura, N., Aguirre, A., James, J.H., Cummings, M.G., Abel, P.M., and Deindoerfer, F. (1974). Plasma amino acids in patients with hepatic encephalopathy: Effects of amino acid infusions. Am. J. Surg., 127, 40-47.

Friedman, E.A., Fastook, J., Beyer, M.M., Rattazzi, T., and Josephson, A.S. (1974). Potassium and nitrogen binding in the human gut by ingested oxidized starch. Trans. Am. Soc. Artif. Intern. Org., 20, 161-167.

Friedman, E.A., Laungani, G.B., Beyer, M.M. (1975). Life prolongation in nephrectomized rats fed oxidized starch and charcoal. Kid. Int., 7, S377-S379.

Friedman, E.A., Saltsman, M.J., Beyer, M.M., and Josephson, A.S. (1976). Combined oxystarch-charcoal trial in uremia: Sorbentinduced reduction in serum cholesterol. Kid. Int., 10, S273-S276.

Friedmen, E.A., Fastook, J., Beyerm, M. M., Ratzanni, T. and Josseph, A. S. (1974). Potassium and nitrogen binding in human guts by ingested oxidised starch. Trans. Am. Soc. Intern. Organs, 20,161-167.

Friedman, EA. (1992).Rationing of uremia therapy, Artificial Organs , Feb.,16(1), 90-97.

Friedman, E.A. and Tao, T.K. (1995). Disappearance of uremia due to heroin-associated nephropathy, American Journal of Kidney Diseases, May, 25(5),689-93

Furukawa, S., Katayama, N., Iizuka, T., Urabe, I., and Okada, H. (1980). Preparation of polyethylene glycol-bound NAD and its application in a model enzyme reactor. FEBS Lett., 121(2), 239-242.

Ganong, W. F. (1994). Review of medical physiology, 408-420, Lange medical publications, California.

Gardner, D.L., Falb, R.D., Kim, B.C., and Emmerling, D.C. (1971). Possible uremic detoxification via oral-ingested microcapsules. Trans. Am. Soc. Artif. Intern. Org., 17, 239-245.

Gardner, D.L., Kjellstrand, C.M., Fink, D.J., and Emmerling, D.C. (1984). Oral administration of microencapsulated urease and zirconium phosphate. Artif.Org., 8, 110-114.

Garofallo, F. and Chang, T.M.S. (1989). Immobilization of *P. picorum* in open pore agar, alginate polylysine alginate for serum cholestrol depletion. J. Biomat., Artifi. Cells and Immo. Biotech., 17, 271-290.

Garofalo, F.A. and Chang, T.M.S. (1991). Effects of mass transfer and reaction kinetics on serum cholesterol depletion rates of free and immobilized *Pseudomonas pictorum*, Applied Biochemistry & Biotechnology, 27(1),75-91.

Gelfand, M.C., Winchester, J.F., Knepshield, J.H., Cohan, S.L., and Schreiner, G.E. (1978). Biochemical correlates of reversal of hepatic coma coated with charcoal hemoperfusion. Trans. Am. Soc. Artif. Intern. Org., 24, 239-242.

Gerlach, J., Jorres, A., Trost, O., Hole, O., Vienken, J., Courtney, J.M., Gahl, G.M., Neuhaus P. (1993). Side effects of hybrid liver support therapy: TNF-alpha liberation in pigs, associated with extracorporeal bioreactors. International Journal of Artificial Organs, Aug., 16(8), 604-608.

Giordano, C. (1980). In: Sorbents and their clinical applications. Academic Press, New York.

Giordano, C., Esposito, R., Randazzo, G., and Pluvio, M. (1972). Oxystarch as a gastrointestinal sorbent in uremia. In Uremia (Kluthe, R., Berlyne, G., and Burton, B., eds.), 231-239, Stuttgart, Thieme.

Giordano, C., Esposito, R., and Pluvio, M. (1975). Oxycellulose and ammonia-treated oxystarch as insoluble polyaldehyde in uremia. Kid. Int., 7, S380-S382.

Giordano, C., Esposito, R., and Pluvio, M. (1976). Further studies with oxystarch. Kid. Int., 10, S266-S268.

Giovannetti, S., and Maggiore, Q. (1964). A low-nitrogen diet with proteins of high biological value for severe chronic uremia. Lancet, 1, 1000-1003.

Goetchus, J. S., and Webster, L. T. Jr., (1965). α-aminobytyrate and hepatic coma. J. Lab. Clin. Med., 65, 257-267.

Goosen, A.M., Mattheus, F.A. (1988). Research on animal cell culture in microcapsules. Chemical engineering education. 22(4) 196-200.

Gordon, A., Greenbaum, M.A., Marantz, L.B., McArthur, M.J., and Maxwell, M.H. (1969). A sorbent based low volume recirculating dialysate system. Trans. Am. Soc. Artif. Intern. Org., 15, 347-352.

Gordon, A., Better, O.S., Greenbaum, M.A., Marantz, L.B., Cral, T., and Maxwell, M.H. (1971). Clinical maintenance hemodialysis with a adsorbent-based, low-volume dialysate regeneration system. Trans. Am. Soc. Artif. Intern. Org., 17, 253-258.

Gregoriadis, G., and Allison, A.C. (1980). Liposomes in Biological Systems. John Wiley & Sons. Chichester.

Groboillot, A., Boadi, D.K., Poncelet, D, Neufeld, R.J. (1995). Immobilization of cells for application in food industry. Critical Review in Biotechnology, 14(2) 75-107.

Grossman, M. J., Wilson, K. and Raper, S. (1993). A novel approach for introducing hepatocytes into portal circulation, J. of Laboratory Clin. Med., 121 (3), 472-478.

Grunwald, J., and Chang, T.M.S. (1979). Continuous recycling of NAD^+ using an immobilized system of collodion microcapsules containing dextran-NAD^+, alcohol dehydrogenase, and malic dehydrogenase. J. Appl. Biochem., 1, 104-114.

Grunwald J., and Chang, T.M.S. (1981). Immobilization of alcohol dehydrogenase, malic dehydrogenase and dextran-NAD^+ within nylon-polyethylenimine micorcapsules: Preparation and cofactor recycling. J. Mole. Catal., 11, 83-90.

Guyton, A. C.(1995). In : Textbook of medical physiology. 9th ed. Philadelphia : W.B. Saunders, Publications, USA.

Hagenfeldt, L., Eriksson, S., and Wahren. J. (1980). Influence of leucine on arterial concentrations and regional exchange of amino acids in healthy subjects. Clin. Sci., 59, 173-181.

Harland, R. C. and Bollinger, R. R., (1994). Extracarporeal hepatic perfusion in the treatment of patient's with acute hepatic failure. Transplan. Rev., 8(2), 73-79.

Hayes, A.W. (1994). In: Principle and methods of toxicology. (Hayes, A.Walace, ed.). Vol. 3,1-17, Raven Press New York.

Herlong, H., and Deihl, A. (1983). BCAA in Hepatic encephalopathy in amino acids : metabolic and medical applications, In: Blackburn, G. et al. (ed.), p 477, Jhon Wright PSG Inc., Boston.

Hikuma, M., Matsuka, H., Kawarai and Karube, I. (1992). Use of microbial electrode for observation of microbiological nitrogen elimination process, Biotech. Bioeng., 40(1), 130-136.

Hughes, R., and Williams, R. (1981). Clinical experience with charcoal and resin hemoperfusion, Seminars in liver disease, 6(2), 169-172.

Ismail, N. and Becker, B.N. (1994). Treatment options and strategies in uremia: current trends and future directions. Seminars in Nephrology, 14(3), 282-299.

Jones, B.D. and Mobley, H.L.T. (1989).*Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. Journal of Bacteriology. Vol.71(12),6414-6422.

Juggi, J. S. (1972). Extracarporeal cation exchange circuits in treatment of hyperammonia liver failure. Med. J., 1, 926.

Juggi, J.S. (1973). Extracorporeal cation-exchange circuits in the treatment of hyperammonemia of liver failure. Med. J. Aust., 1, 926-930.

Kasai, Shin-ichi, Sawa, M., and Mito, M. (1994). Is the biological artificial liver clinically applicable ? A Historic review of biological artificial liver support systems. Artif. Organs, 18(5): 347-354.

Katayama, N., Urabe, I., and Okada, H. (1983). Steady-state kinetics of coupled two-enzyme reactor with recycling of poly(ethylene glycol)-bound NAD^+. Eur. J. Biochem., 132, 403-409.

Kato, T., Berger, S.J., Carter, J.A., and Lowry, O.H. (1973). An enzymatic cycling method for nicotinamide-adenine dinucleotide with malic and alcohol dehydrogenase. Anal. Biochem., 53, 86-97.

Kennedy, J.F. (1987). Biotechnology. Vol.7a, VCH Publishers, New York.

Kieley, J. E., Pender, J. C., Welch, H. F., and Welch, C. S. (1958). Ammonia intoxication treated by hemodialysis. New. Engl. J. Med., 256, 720-726.

Kim, S.D., and Spizizen, J. (1985). Molecular cloning and expression of *B. pasteurii* urease gene in *E. coli,* K. J. of Microb. Bioeng., 13, 297-302

Kitajima, M., and Kondo, A. (1971). Fermentation without multiplication of cells using microcapsules that contain zymase complex and muscle enzyme extract. Bull. Chem. Soc. Japan., 44(11), 3201-3202.

Kitano, H., and Ise, N. (1984). Hollow fiber enzyme reactors. Trends in Biotech., 2, 5-7.

Kjellstrand, C., Borges, H., Pru, C., Gardner, D., and Fink, D. (1981). On the clinical use of microencapsulated zirconium phosphate-urease for the treatment of chronic uremia. Trans. Am. Soc. Artif. Intern. Org., 27, 24-30.

Klebonoff, G., Hollander, D., and Cosimi, A. B. (1972). Asonguineous hyperthermic total body perfusion (T.B.W.) in treatment of stage IV hepatic coma. J. Surg. Res., 12:1.

Klinkmann H., Falkenhagen, D. Courtney, J.M. (1979). Blood purification by hemoperfusion. International Journal of Artificial Organs 2(6),296-308.

Knell, A. J. (1981). Hemodyalysis procedure in acute liver failure. In: Artificial liver support, Brunner and Schmidt (ed.), 147-152. Springer-Verlag, NY.

Knell, A. J. (1981). Hemodyalysis procedure in acute liver failure. In: Artificial liver support in Brunner and Schmidt (ed.), 147-152. Springer-Verlag, NY.

Kopple, J.D. (1978). Abnormal amino acid and protein metabolism. Kid. Int., 14, 340-348.

Krebs, H. A. Hems, R., Lund, P., Holiday, D., and Read, W. C. (1978). Sources of ammonia for mammalian urea synthesis. Biochem. J. 176: 733-737

Kuster, G. G. R. (1976). Hepatic support in acute liver failure. Springfield, Ill, Charles C. Thomas, Publisher.

Langer, R., and Vacanti, J. P.(1993). Tissue engineering. Science, 260, 920-926.

Ladish et al. (1992). Putting biotechnology to work: bioprocess engineering, Committee on bioprocess engineering, National Research Council (Ladish, Chairman), Washington, DC.

Lee, C., and Tink, A. (1958). Exchange transfusion in hepatic coma. Case report, Med. J. Aust.,1, 40-41.

Lehninger, A.L. (1982). Principles of biochemistry. Worth Publishers, INC., New York.

Lim, F., and Sun, A.M. (1980). Microencapsulated islets as bioartificial endocrine pancreas. Science, 210, 908-910.

Lloyd-George, I. and Chang, T.M.S. (1993). Free and microencapsulated *Erwinia herbicola* for the production of tyrosine, Biomaterials, Artificial Cells, & Immobilization Biotechnology, 21(3), 323-333.

Lloyed-George, I.(1993). The conversion of ammonia, phenol and pyruvate into tyrosine by the use of the tyrosine phenyl lyase activity of microencapsulated *Erwinia herbicola*, Ph.D. thesis,page-127, McGill University, Montreal, Canada.

Lloyd-George, I., and Chang, T.M.S. (1995). Charecterization of free and APA microcapsulated *Erwinia herbicola* for the conversion of ammonia, pyruvate, and phenol into L-tyrosine. Biotechnology & Bioengineering, 48(6),706-714.

Lowe, C.R. (1981). Immobilized coenzymes. In topics in enzyme and fermentation biotechnology (Wiseman, A., ed.), l5, 13-146, John Wiley & Sons, New York.

Lowe, C.R., and Dean, P.D.G. (1971). Affinity chromatography of enzymes on insolubilized cofactors. FEBS Lett., 14(5), 313-316.

Malchesky, M. (1994). Nonbiological liver support: history overview. Artif. Organs. 18(5), 342-347.

Malchesky, P. S. (1972). Further assessment of liver tissue materials for extracorporeal hepatic assist. In : Williams et el. (eds.) Pitman Medical London, p 202.

Man, N-K., Drueke, T., Becker, A., Zingraff, J., Jungers, P., and Brentano, J.L.F. (1976). Clinical use of oxystarch. Kid. Int., 10, S269-S272.

Mathew, J.T., and Hausinger, R.P.(1987). Purification and characterisation of Ni containing multicomponent urease from *K. aerogenes*, J. of Biol. Chem., 262(13), 5963-5967.

Manecke, G. (1974). Immobilized enzymes. Chimia, 28, 467-474.

Mansson, M.O., Larsson, P.O., and Mosbach, K. (1978). Covalent binding of an NAD analogue to liver alcohol dehydrogenase resulting in an enzyme-coenzyme complex not requiring exogenous coenzymes for activity. Eur. J. Biochem., 86, 455-463.

Mansson, M.O., and Mosbach, K. (1987). Immobilized active coenzymes. In Methods in Enzymology (Mosbach, K., ed.), Vol.136, 3-9, Academic Press, New York.

Mara, D. D. and Haward, P. (1986). Artificial fresh water environment: waste stabilisation ponds. In: Biotechnology, volume on microbial degradation. Rehm, H.J. and Reed, G.,(eds.),8,179-206.

Martin, I. and Courtney, J.M. (1977). Oral adsorbents in the treatment of uremia: a review. Journal of Medical Engineering & Technology, Mar.,1(2), 92-110.

May, S.W., and Li, N.N. (1972). The immobilization of urease using liquid-surfactant membranes. Biochem. Biophys. Res. Commun., 47, 1179-1185.

May, S.W., and Li, N.N. (1977). Liquid-membrane-encapsulated enzymes. In Biomedical Applications of Immobilized Enzymes and Proteins (Chang, T.M.S., ed.), Vol.1, 171-190, Plenum Press, New York.

McDermott, W.V., Jr., and Adama, R.D. (1954). Episodic stupor associated with an Eck fistula in the human with particular reference to the metabolism of ammonia. J. Clin. Invest., 33, 1-9.

Meriwether, L.S., and Kramer, H.M. (1976). In-vitro reactivity of oxystarch and oxycellulose. Kid. Int., 10, S259-S265.

Messing, R.A. (1975). Immobilized Enzymes for Industrial Reactors. Academic Press, N New York.

Minana, M.D., Marcaida, G., Grosolia, S., and Felipo, V. (1995). Prenatal exposure of rats to ammonia impairs NMDA receptor function and affords delayed protection against ammonia toxicity and glutamate neurotoxicity. J. of Neuropathology and Experimental Neurology, 54(1), 644-650.

Mitch, W.E. (1983). Amino acid analogues: Metabolism and use in patients with chronic renal failure. In Amino Acids: Metabolism and Medical Applications (Blackburn, G.L., Grant, J.P., and Young, V.R., eds.),439-450, John Wright-PSG Inc, Boston.

Mito, M. (1986). Hepatic assist-present and future. Artificial Organs, 10, 214-218.

Mobed, M. and Chang, T.M.S. (1991). Preparation and surface charecterization of carboxymethylechitin incorporated submicron bilayer lipid membrane artificial cells. J Biomat. Artif. Cells and Imm. Biotech., 19, 731-744.

Mobed, M. and Chang, T.M.S. (1993). Purification and quantitative determination of carboxymethylechitin incorporated submicron bilayer lipid membrane artificial cells (liposomes) encapsulating haemoglobin. J. Biomat. Artif. Cells and Imm. Biotech., 20, 329-336.

Mobley, H.L.T., Hausinger, R. P. (1989). Microbial ureases: significance, regulation, and molecular characterization. Microbiological Reviews. Vol. 53, 85-108.

Mobley, H.L.T., Michael, D.I., Hausinger, R. (1995). Molecular biology of microbial ureases, Microbiological Review, 59(3), 451-480.

Mohsini, K., Lister, C., and Chang, T.M.S. (1980). The effects of homologous cross circulation and in situ liver perfusion on Galactosamine induced fulminant hepatic rat rats. Artif. Organs., 4, 171-175.

Moo-Young, M. (1985). Comprehensive Biotechnology. Vols.1-4, Pergamon Press, New York.

Mosbach, K., (1976). Methods in Enzymology. Vol.44, Academic Press, New York.

Mosbach, K. (1987). Methods in Enzymology. Vols.135-136, Academic Press, New York.

Mosbach, K. (1988). Methods in Enzymology. Vol.137, Academic Press, New York.

Mosbach, K., and Mosbach, R. (1966). Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their applications in column techniques. Acta Chem. Scand., 20, 2807-2810.

Mosbach, K., Larsson, P.O., and Lowe, C. (1976). Immobilized coenzymes. In Methods in Enzymology (Mosbach, K., ed.), 44, 859-887, Academic Press, New York.

Mudarak, K., and Kunst, Sabine (1988). Elimination of nitrogen and phosphorus by microorganisms. In Biotechnology, volume on special microbial processes. Rehm, H.J. and Reed, G. (eds.), 6b, 437-462.

Mueller, P., and Rudin, D.O. (1968). Resting and action potentials in experimental bimolecular lipid membranes. J. Theor. Biol., 18, 222-258.

NIH Census Statement (1993), Nov. 11(2),1-3.

Mulrooney, S.B., Lynch, M.J. Mobley, H.L.T. and Hausinger, R.P. (1988). Purification, characterization, and genetic organization of recombinant *Providencia stuartii* urease expressed by *Escherichia coli*. Journal of Bacteriology. 170(5):2202-2207.

Mulrooney, S.B., Pankratz, H. S., and Hausinger, R. P. (1989). Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes* urease. Journal of General Microbiology. v. 135, p. 1769-1776.

Nealon, T.F.,Jr., Ching, N. (1962). An extracorporeal device to lower blood ammonia levels in hepatic coma. Trans. Am. Soc. Artif. Intern. Org., 8, 226-228.

Nealon, T., F., Ching, N.P.H. (1964). Ion exchange resin in hepatic coma. Biochem. Clin., 3, 263-265.

Neuzil et al. (1993). Use of novel bioartificial liver by in a patients with acute liver insufficiency. Surgery, 113, 340-343.

Nose, Y., Koshino, I., Castino, F., Yoshida, K., Carse, Kretz, A. P., and Nose, Y. (1989). Artificial liver for a bridge to liver transplantation. Artif. Org., 13, 415-416.

Oberley, M.A., and Neal R. Glass, M.D., Charles C. Thomas Publishers, 2600 South First Street, Springfield, IL 62794-9265

Oberley, T., and Terry, D. O. (1987). Understanding Kidney Transplantation. Trans. Am. Soc. of Artifi. Int. Org., 35, 570-572.

Oberley, T., and Terry D. Oberley, (1991). *Your* New Life With Dialysis - A Patient Guide for Physical and Psychological Adjustment .Charles C. Thomas Publishers. 2600 South First Street. Springfield, IL 62794-9265.

Okada, M., Tabata, Y., Kobayashi, H., Nomura, Y., Soma, H., Hirasawa, H., and Sato, H. (1978). Clinical experience of bead-shaped charcoal hemoperfusion in chronic renal failure and fulminant hepatic failure. In Artificial Kidney, Artificial Liver, and Artificial Cells (Chang, T.M.S., ed.),79-88, Plenum Press, New York.

Orten, J.M., and Neuhaus, O.W. (1975). Human Biochemistry. 9th Edition, The C.V. Mosby Company, Saint Louis.

Ostergard, J.C.W., and Martiny, S.C. (1973). The immobilization of beta-galactosidase through encapsulation in water-insoluble microcapsules. Biotechnol. Bioeng., 15, 561-563.

Parfrey, P.S. (1994). Clinical epidemiology in chronic uremia. Clinical & Investigative Medicine - Medecine Clinique et Experimentale, Oct.,17(5), 466-473.

Pearson, H.W., Mara, D.D., and Arridge H.A. (1995). the influence of pond geometry and configuration on facultative and maturation waste stabilization pond performance and efficiency. Water Science & Technology. 31(12):129-139.

Piorry quoted by Fishberg, A. M. (1931). In : Hypertension and nephritis. 2nd ed. Lea and Febiger (ed.). p. 619.

Piskin, Erthan (1984). Semipermeable membrane suitable for hemoperfusion (Chang, T, M. S. and Bing-Lin, H. (eds.). Proceedings of International Symposium on Hemoperfusion and Artificial Organs. Nov. 20-22, China academic publishers, Beijing, P.R. of China.

Poznansky, M.J., and Chang, T..M.S. (1974). Comparison of the enzyme kinetics and immunological properties of catalase immobilized by microencapsulating and catalase in free solution for enzyme replacement. Biochem. Biophy. Acta, 334, 103-115.

Pye, E.K., and Wingard,L.B.Jr. (1974). Enzyme Engineering. Vol.2, Plenum Press, New York.

Pye, E.K., and Weetall, H.H. (1978). Enzyme Engineering. Vol.3, Plenum Press, New York.

Raaby, W. A. (1989). Neurophysiology of ammonia intoxication (Butherworth, R. F., et al. ,eds.), Hepatic encephalopathy: Pathophysiology and treatment. Humana Press, 49-77.

Record, C. O., Chase, R. A., Curzon, G. and Murray-Lyon, and Iain, M. (1975). Amino acid changes in hepatic encephalopathy and their relationship to disturbed cerebral neurotransmission, (Williams, R. and Murray-Lyon, Iain, M., ed.) Artificial liver support. 27-30, Pitman medical Publ. Kent.

Ritchi, H. D., Devise, D. M., et al. (1962). An extracorporeal methods for reducing high blood ammonia levels. Gut, 3:172.

Robert, S.M., Turner, N.J., Willetts, A.J., and Turener, M.K. (1995). Introduction to biocatalysis using enzyme and microorganisms, 1-15, Cambridge university press.

Rony, P.R. (1971). Multiphase catalysis: II. Hollow fiber catalysts. Biotech. and Bioeng., 13, 431-447.

Rosen, H.M., Yoshimura, N., Hodgman, J.M., and Fischer, J.E. (1977). Plasma amino acid patterns in hepatic encephalopathy of differing etiology. Gastroenterology, 72,483-487.

Rosenbaum, J. L.(1978). Experience with resin hemoperfusion. (Chang, T.M.S., ed.). Artificial Kidney, Artificial Liver and Artificial Cells. Plenum Press, New York.

Rosenbaum, J.L., Winsten, S., Kramer, M.S., Moros, J., and Raja, R. (1970). Resin hemoperfusion in the treatment of drug intoxication. Trans. Am. Soc. Artif. Intern. Org., 16, 134-140.

Rozga, J. (1993). Development of bioartificial liver : Properties and functions of a hallow fibre module inoculated with liver cells. Hepatology, 17-258-265.

Rozga, J. (1994). A bioartfificial liver to treat sever acute liver failure. Annals of Surgery, 219(5), 538-546.

Ryan, Klinkmann H., Falkenhagen, D., and Courtney, J.M. (1979). Blood purification by hemoperfusion. International Journal of Artificial Organs, 2(6),296-308.

Ryan, C.J., Courtney, J.M., Wood, C.B. Hood, and R.G., Blumgart, L.H. (1979). Activated charcoal hemoperfusion via an extracorporeal circuit in the unrestrained and unanaesthetized rat. British J. of Experimental Pathology, 60(4),400-410.

Ryan, C.J., Courtney, J.M., McLay, A.L., Wood, C.B., and Blumgart, L.H. (1982). The effect of repeated hemoperfusion over charcoal in the conscious rat on liver Pathophysiology. Artificial Organs, 6(2), 169-174.

Schechter, G. E. (1958). A simple extracorporeal device for reducing blood ammonia levels. Sugery, 44, 892-893.

Schiff, L. (1975). Diseases of the Liver. 4th Edition, J. B. Lippincott Company, Philadelphia.

Schreiner, G.E. and Maher, J.F. (1929). In : Uremia : biochemistry, pathogenesis and treatment.

Schuber, F., and Travo, P. (1976). Calf-spleen nicotinamide- adenine dinucleotide glycohydrolase: Solubilization purification and properties of the enzyme. Eur. J. Biochem., 65, 247-255.

Sefton, M.V. and Nishimura, E. (1980). Insulin permeability of hydrophilic polyacrylate membranes. Journal of Pharmaceutical Sciences, 69(2), 208-209.

Setala, K., Heinonen, H., and Schreck-Purla (1972). Uremic waste recovery. Proc. Conf. EDTA, 9, 514-520.

Setala, K., Heinonen, H., and Schreck-Purla (1973). Ingestion of lyophilized soil bacteria for alleviation of uremic symptoms. J. Int. Res. Comm. Syst., 73.

Shasul, G. M. (1986). Parathyroid hormone : A uremic toxin. In: Uremic toxins, Severin, R., Raymond, V. Shaul, G. M., eds., Plenium press, NewYork, 223,1-15.

Sherlock, S. (1989). Diseases of liver and bilary system, 80-115, 8 th edn. Blackwell Sci. Publi. London.

Shi, Z.Q., and Chang, T.M.S. (1982). The effects of hemoperfusion using coated charcoal or tyrosinase artificial cells on middle molecules and tyrosine in brain and serum of hepatic coma rats. Trans. Am. Soc. Artif. Intern., Org., 28, 205-209.

Shi, Z. Q., and Chang, T.M.S. (1984). Amino acid disturbances in experimental hepatic coma rats, Int. J. Artifi. Organs, 7, 197-202.

Shoon-Shiang, P., Feldman, E., Nelson, R., Heintz, R., Yao, Q., Yao, Z.W., Zheng, T.L., Merideth, N., Skjakabraek, G., Spevik, T., Smidsrod, O., and Sandford, P. (1993). Long-term reversal of diabetes by the injection of the immunoisolated islets. Proc. Nat. Acad. Sci. USA , 90, 5843-5847.

Shu, C.D., and Chang, T.M.S. (1980). Tyrosinase immobilized within artificial cells for detoxification in liver failure: I. Preparation and in-vitro studies. Int. J. Artif. Org., 3, 287-291.

Shu, C.D., and Chang, T.M.S. (1981). Tyrosinase immobilized within artificial cells for detoxification in liver failure: II. In-vivo studies in fulminant hepatic failure rats. Int. J. Artif. Org., 4, 82-85.

Siu Chong, E.D., and Chang, T.M.S. (1974). In-vivo effects of intraperitoneally injected L-asparaginase solution and L-asparaginase immobilized within semipermeable nylon microcapsules with emphasis on blood L-asparaginase, body L-asparaginase, and plasma L-asparagine levels. Enzyme, 18, 218-239.

Snapper, I., and Schaeffer, I. E. (1958). Treatment of two patients with hepatorenal syndrome and acute renal failure by asanguinotrnsfusion. Ann. Intern. Med., 34,692.

Sparks, R.E., Salemme, R.M., Meier, P.M., Litt, M.H., and Lindan, O. (1969). Removal of waste metabolites in uremia by microencapsulated reactants. Trans. Am. Soc. Artif. Intern. Org., 15, 353-358.

Sparks, R.E., Mason, N.S., Meier, P.M., Litt, M.H., and Lindan, O. (1971). Removal of uremic waste metabolites from the intestinal tract by encapsulated carbon and oxidized starch. Trans. Am. Soc. Artif. Intern. Org., 17, 229-235.

Starzl, T. E., Marchiaro, T. L., and Huntley, R. T. (1966). Experimental and clinical homotransplantation of liver, with particular reference to hepatic supportive procedures, antilymphocytes serum, and tissue typing. Am. J. of Surg., 112, 391-3.

Stefoni, S., Feliciangeli, G., Coli, L, Prandini, R. Bonomini, V., Use of combined (1982). hemodialysis/hemoperfusion in chronic uremia, Contributions to Nephrology, 29, 123-132.

Stevenson, W.T.K., Evangelista, R.A., Sugamori, M.E., and Sefton, M.V. (1988). Microencapsulation of mammalian cells in a hychoxyethyl methacrylate-methyl methacrylate copolymer: Preliminary development. Biomat. Art. Cells and Art. Org.,16, 747-769.

Sugamori, M., Boag, A.H., Broughton, R.L., and Sefton, M.V. (1985). Microencapsulation of mammalian cells in polyacrylate membranes for metabolic prostheses. In Progress in Artificial Organs (Nose, Y., Kjellstrand, C., and Ivanovich, P., eds.), 630-634, ISAO Press, Cleveland.

Sun, A.M. O'Shea, G. Van Rooy, H. Goosen, M. F. (1982).Microencapsulation of the islands of Langerhans and the artificial pancreas. French Journees Annuelles de Diabetologie de l Hotel-Dieu., 161-168.

Sun, A. M., Cai, Z., Shi, Z., OShea, G. M. (1986). Microencapsulated hepatocytes as bioartifficial liver. Trans. Am. Soc. Artif. Intern. Organs., 32, 39-41.

Sundaram, P.V., Pye, E.K., Chang, T.M.S., Edwards, V.H., Humphrey, A.E., Kaplan, N.O., Katchalski, E., Levin, Y., Lilly, M.D., Manecke, G., Mosbach, K., Patchornik, A., Porath, J., Weetall, H.H., and Wingard, L.B., Jr. (1972). Recommendation for standardization of nomenclature in enzyme technology. In: Enzyme Engineering (Symposium No.3 of Biotechnology and Bioengineering, Wingard,L.B.Jr., ed.), 15-18, John Wiley & Sons, New York.

Takahasi, T., Malcheky, P., and Noyes, Y. (1991). In: Artificial liver : state of art, digestive diseases and science, 36(9), 1327-1340.

Takamiya, S., Ohshima, T., Tanizawa, K., and Soda. K. (1983). A spectrophotometric method for the determination of aminopeptidase activity with leucine dehydrogenase. Anal. Biochem., 130(1), 266-270.

Thomason, J.M., Seymour, R.A. and Ellis, J. (1994). The periodontal problems and management of the renal transplant patient. Renal Failure, Nov., 16(6), 731-45

Townsend, W.B., Courtney, J.M. Gilchrist, T.(1978). Activated carbon for hemoperfusion. Int. Journal of Artifi. Org., 1(3), 148-50.

Trevan, M.D. and Baffey, S., Goulding, K.H., and Stansbury, P. (1987). Biotechnology the basic principle. 1-13, Open university press, New York.

Trey, C., and Davidson, C. S. (1970). In: The management of fulminant hepatic failure, Proper, H.,ed., Vol. 3, 282-293, Grune and Stratton, New York.

Uchino, J., and Matsushita, M. (1994). Artificial Liver : Strategies for rescue of patients with liver failure. ASAIO Journal, 40(1), 74-77.

Vieth, W.R., Venkatasubramanian, K., and Constantinides, A. (1979). Biochemical Engineering. The New York Academy of Sciences, New York.

Vorlop, K.D., and Klien, J. (1985). Immobilizations techniques-cells. Principle of Biotechnology, 203-224.

Walker, C. O., and Schenker, S. (1970). Pathogenesis of hepatic encephalopathy with special reference to the role of ammonia. Am. J. Clin. Nutr., 23, 619-632.

Walser, M. (1978). Keto acid therapy in chronic renal failure. Nephron, 21, 57-74.

Wandrey, C. (1985). Coenzyme regeneration in membrane reactors. Biotechnol. Ser., 5, 177-208.

Weil-Malherbe, H. (1958). The metabolism of ammonia in brain in consciousness and the chemical environment of the brain. 25th Ross Paediatric research Conference, p.50.

Williams, R., and Murray-Lyon, I.M. (1975). Artifi. Liver. Support. Pitman Medical, London.

Wingard, L.B. Jr., Berezin, I.V., and Klyosov, A.A. (1980). Enzyme Engineering: Future Directions. Plenum Press, New York.

Wingard, L.B.Jr., Katchalski-Katzir, E., and Goldstein, L. (1976). Appl. Bioch. and Bioeng.Vol.1 (Immobilized Enzyme Principles), Academic Press, New York.

Winchester, J.F. (1982). Potential for hemoperfusion in uremia. Contributions to Nephrology, 29, 114-122.

Wolpert, E., Philips, S. F, and Summerskill, W. H. J. (1971). Transport of urea and ammonia production in human colon. Lancet, (2), 1387-1390.

Wong, H., and Chang, T.M.S. (1986). Bioartificial liver: Artificial cells microencapsulated hepatocytes increases survival of liver failure rats. Int. J. Artif. Org., 9, 335-336.

Wong. H., and Chang, T.M.S. (1988). The viability and regeneration of artificial cell microencapsulated rat hepatocytes xenograft transplants in mice. Biomat. Art. Cells Art. Org., 16, 731-739.

Wong. H., and Chang, T.M.S. (1988). A novel two step procedure for living cell microecapsules for improving xenograph servival. J. Biomat. Artifi. Cells and Immo. Biotech., 19, 687-698.

Xing, X.H., Shiragami, N., and Unno, H. (1995). J. of Chem. Eng. of Japan, 28(5), 525-530.

Yangi, K., Ookawa, K., Mizuno, S., Ohshima, N. and Yagi, K. (1989). Performance of a new hybrid artificial liver support system using hepatocytes entrapped within a hydrogel. Trans. Am. Soc. of Artifi. Int. Organs, 35, 570-572.

Yu, Y.T., and Chang, T.M.S. (1982). Immobilization of multienzymes and cofactors within lipid-polyamide membrane microcapsules for the multistep conversion of lipophilic and lipophobic substrates. Enzyme Microb. Technol., 4, 327-331.

Yuan, Z.Y., and Chang, T.M.S. (1986). Rat microsomes and cytosol immobilized by microencapsulation in artificial cells. Int. J. Art. Org., 9, 63-68.

Zaborsky, O.R. (1973), Immobilized Enzymes, CRE Press, Cleveland, Ohio.

Zaki, A. E. O., Eardel, E. N., and Canalese, J. (1988). Potential toxins of acute liver failure and their effects on blood brain barrier permeability. Experimentica, 39, 988-991.

Zeive, L., (1975). Metabolic abnormalities in hepatic coma and potential toxin to be removed In: Willims, R., Murrey, L. (ed.), Artificial liver support, 11-26, Pitman medical Publ. Kent.

Zeive, L., Doziaki, W. M., and Zeive, F. J. (1974). Synergism between mercaptanes and ammonia or fatty acids in production of coma, J. Lab. Clin. Med., 83, 18-28.

Zieve, L. (1982). Hepatic encephalopathy, 433-459, In : Popper, H. and Schiff, E. R. (ed.), Lipinocott co. Philadelphia.

Zieve, L. (1979). Amino acids in liver failure. Gastroenterology, 76, 219-221.

Zink, M.W., and Sanwal, B.D. (1962). The distribution of substrate specificity of L-leucine dehydrogenase. Arch. Biochem. Biophys., 99, 72-77.

Zkim, D., and Boyer, T. (1990). In : Hepatology; A text book of liver diseases, 1,447-456, Saunders, W. B. Co., Philadelphia.

PREFACE FOR CHAPTER 2 TO 7

To evaluate the feasibility of the novel approach of using artificial cells containing genetically bacterial cells for various applications, artificial cells containing genetically engineered *E. coli* DH 5 cells were prepared. **Chapter 2** describes the unique artificial cell system for the removal of urea and ammonia from a reaction media. This system was chosen as presently we do not have a suitable urea and ammonia removal system. Also, to establish a precedent of feasibility of this new hypothesis of using artificial cells having other types of genetically engineered bacterial cells for the future applications.

Based on the studies of **Chapter 2**, I proposed and developed three approaches for various potential applications. The first approach involves the use of batch reactor, the second entails the use of a column bioreactor, and the third is an in-vivo animal study for the removal of urea and ammonia. In **Chapter 3** the details of the batch reactor study for the removal of urea and ammonia from the plasma is described. In addition, the comparison of urea and ammonia removal kinetics from plasma and reaction media along with a detailed study of optimisation of the process parameters for the bacterial cell immobilization by microencapsulation is discussed. In **Chapter 4**, I describe the use of column bioreactors containing artificial cells for the removal of plasma urea and ammonia.

Chapter 5 is an overview of the in-vitro studies including preliminary results of studies on uremic rat models. For the animal study, I designed a new type of experimental uremic rat model. In, **Chapter 7**, specific details of plasma urea removal in an experimental rat model and microcapsule morphology study is described. **Chapter 8** contains the details of the in-vivo results involving uremic rat models. A detailed comparative study for the urea and ammonia efficiency of this new approach with the existing methods is also presented in this chapter.

In brief, the detailed studies for each system will be presented in **Chapters 2 to 7**.

The results obtained in my research have been presented in the following papers:

Research articles :

1. S. Prakash and T.M.S. Chang (1993). Genetically engineered *E. coli* cells, containing *K. aerogens* genes, microencapsulated in artificial cells for urea and ammonia removal : Biomaterials Artificial Cells and Immobilization Biotechnology, Vol. 21 (5), 629-636 p.p. (Marcel Dekker Inc. New York).
2. S. Prakash and T.M.S. Chang (1995). Preparation and in-vitro analysis of genetically engineered *E. coli* DH5 cells, microencapsulated in artificial cells for urea and ammonia removal : Biotechnology and Bioengineering, Vol. 46(6), 621-626 p.p, John Willy and Sons, USA.
3. S. Prakash and T.M.S. Chang (1996). Artificial cells for plasma urea and ammonia removal using continuous process column bioreactor having genetically engineered *E. coli* DH5 cells. Biomaterials Artificial Cells and Immobilization Biotechnology, an International Journal. Accepted along with other results.
4. S. Prakash and T.M.S. Chang (1996). A new approach of using artificial cells containing genetically engineered *E. coli* DH5 cells for urea and ammonia removals. Submitted to ASAIO journal.
5. S. Prakash and T.M.S. Chang (1996). Microencapsulated genetically engineered live *E coli* DH5 cells administered orally to maintain normal plasma urea level in uremic rats. Submitted to Nature.
6. S. Prakash and T.M.S. Chang (1996). Microencapsulated genetically engineered *E. coli* DH5 cells for the control of urea and ammonia by simple oral feeding in experimental uremic rat models. Manuscript in preparation.
7. S. Prakash and T.M.S. Chang (1995). Microencapsulated genetically engineered *E. coli* cells for urea and ammonia removal in kidney failure and Liver failure: IEEE Transaction in Biomedical engineering (in Press).

Book chapter :

1. T. M. S. Chang and S. Prakash (1995). Artificial cells for bioencapsulation of cells and genetically engineered *E. coli* : For cell therapy, gene therapy and removal of urea and ammonia, In : Methods in Molecular Biology, Volume on "Expression and Detection of Recombinant Gene". Humana Press, USA Chapter 75 (in press).

Abstract :

1. S. Prakash and T.M.S. Chang (1995). Microencapsulated genetically engineered *E. coli* cells for urea and ammonia removal in kidney failure and liver failure: IEEE transaction in Biomedical engineering,, EMBS 95, Montreal, Canada.
2. S. Prakash and T.M.S. Chang (1994). Genetically Engineered *E. coli* DH 5 cells, containing *K. aerogens* genes, microencapsulated in artificial cells for plasma urea and ammonia removal : XI congress of the Society of the Artificial Cells, Blood Substitute and Immobilization Biotechnology organized by Massachusetts Institute of Technology, Boston, USA, July 24.
3. S. Prakash and T.M.S. Chang (1994). Genetically engineered *E. coli* cells, containing *K. aerogens* genes, microencapsulated in artificial cells for the diseased state plasma urea and ammonia removal: China-Canada Biotechnology Conference, Tianjin, Peoples Republic of China, May 8-11, 28.

In accordance with McGill University regulations, six of the above manuscripts (the major publications 1 to 6) are reproduced in their original form in full as individual chapters **(Chapters 2 to 7)**. Details not reported in the original manuscripts are added as appendices.

CHAPTER 2

Genetically Engineered *E. coli* Cells, containing *K. aerogen* gene, Microencapsulated in Artificial Cells for Urea and Ammonia Removal

S. Prakash, and T.M.S. Chang

Biomaterials Artificial Cells and Immobilization Biotechnology;
Vol. 21, page 629-636 (1993)

2.1 ABSTRACT

Microencapsulated genetically engineered *Escherichia coli* cells can efficiently remove urea without an increase of the ammonia in the medium. A quantity of 100 mg alginate poly-L-lysine alginate (APA) encapsulated bacteria rapidly reduces urea in a 100 ml solution. The original urea concentration 100.00 ±1.00 mg / dl falls to 1.55 ± 0.13 mg / dl in 30 minutes, with no increase of ammonia in the reaction medium. Extrapolated results show that the urea depletion capacity of encapsulated bacteria is sufficient to remove urea during kidney failure. Using a single pool model, 40.00 g of encapsulated genetically engineered *E. coli* can lower urea (100 mg / dl) from 40 liters of the body fluid compartment to 1.60 mg /dl within 30 minutes. Also, 40.00 g of encapsulated bacteria can lower ammonia (758.00 μM/L), in 40 liters of body water, to 90.42 μM/L in 20 minutes. Further studies will be required for multi-compartmental models in the physiological conditions.

2.2 INTRODUCTION

There is marked elevation of systemic urea in kidney failure and ammonia in liver failure. In other diseased states, there is also an elevated level of urea and ammonia. Haemodialysis can remove excessive urea in renal failure. However, a more efficient urea removal system is required for hemoperfusion and other approaches in renal and liver failure. Extensive research has been carried out by different researchers on urea removal (Esposito and Giordano, 1980). Despite this no system with sufficient urea removal capacity is yet available. One approach amongst them is the use of microencapsulated enzyme urease in converting urea into ammonia which is then removed by an adsorbent (Chang, 1966). This was based on the concept and principle of artificial cells (Chang,

1962). This led to clinical trial of micrencapsulated zirconium phosphate-urease on uraemic patients (Kjellstrand et al.,1981). The clinical results were encouraging, but this system requires a very large amount of microcapsules. This is because of the low capacity of the ammonia adsorbents. The other approach was the use of oxystarch (Esposito and Giordano, 1980). Again, clinical results were promising but required a large dosage of oxystarch and its low urea removal efficiency at neutral pH was the problem. The lyophilized urea utilizing soil bacterial cells were also not successful (Sitala et al., 1973). In another approach, an encapsulated multienzyme system that converts urea and ammonia into essential amino acids was successfully demonstrated (Gu and Chang, 1990). However, multienzyme systems require a better cofactor recycling system to give a sufficient rate of urea and ammonia conversion.

Urea is used by a wide variety of bacteria, fungi, and plants (Sitala et al., 1973; Stephen et al., 1988; Mobley and Haussinger, 1989). Earlier studies using lyophilized urea utilizing soil bacterial cells were not successful because the bacteria lacked sufficient urea utilization rate. With the recent development of genetic engineering, effective bacterial cells are now available. In the nitrogen limiting condition, normal *E. coli* uses urea as a nitrogen source (Mobley and Haussinger, 1989; Mulrooney et al., 1988; Mulrooney and Haussinger 1989). The urea utilization capacity of this bacteria is enhanced by genetic engineering (Mulrooney et al. 1988; Mulrooney and Haussinger 1989). The method of microencapsulation of biologicals (Chang, 1965) is now well established and is in use for biomedical applications. Reviews are available (Chang, 1988; Chang,1991; Chang, 1992).

This paper presents a new approach for urea and ammonia removal based on the use of genetically engineered *E. coli* cells which uses urea directly. This is based on the microencapsulation concept and the use of urea inducible urea utilizing capacity of genetically engineered *E. coli* cells which contains *K. aerogenes* gene.

2.3 MATERIALS AND METHODS

2.3.1. Microorganism and Culture Media :

Bacteria *E. coli* DH5 strain was a generous gift from Prof. R. P. Hausinger (Mulrooney et al., 1989). The Luria Bertanica (L. B.) growth media was used for primary cell cultivation (Maniatis et al., 1991). The L. B. growth medium was comprised of bacto tryptone (Difco) 10.00 g, bacto yeast extract (Difco) 5.00 g, and sodium chloride (Sigma) 10.00 g per litre. The pH of the media was adjusted to 7.5 by adding suitable amounts of 1 N NaOH. Media and other solutions were sterilized in Castele Labclaves for 30 min. at 250 ^{0} F.

2.3.2 Chemicals :

Alginic acid (low viscosity) and Poly-L-lysine (mol. weight 16,100) are from Sigma chemicals Co., USA. All other chemicals were of analytical grades.

2.3.3. Microencapsulation :

Microcapsules containing bacterium *E. coli* were prepared. The microencapsulation procedure used was a modification of the procedure reported earlier from this center (Wong and Chang, 1991). Briefly, this was done by allowing droplets of bacterial cells, suspended in autoclaved sodium alginate solution in 0.9% sodium chloride, to fall into an autoclaved aqueous solution of ice cold calcium chloride. The viscous solution was pressed through syringe (23 gauge) by syringe pump (Compact infusion pump model-975 Harward App. Co. Inc. Mass.). The compressed air was passed through another syringe (16 gauge) by air pump to cut the droplets coming out of the

syringe pump. The combined of these two needles are called as droplet needle assembly. The droplets were allowed to gel for 15 minutes in gentle stirring solution of calcium chloride. The microcapsules were then washed ten times with 0.9 % sodium chloride and stored at 4^0 C. The conditions were kept sterile during the process of microencapsulation.

2.3.4 Reaction Media for Urea Removal Experiments :

Reaction media comprised of glucose 1.00 g, magnesium sulfate 20.00 mg, dipotassium monohydrogen phosphate 30.00 mg, and vitamin B_{12} 0.07 g per litre was used during the experimentation. When required, filtered sterilized urea was added into the reaction media.

2.3.5. Experimental Procedure :

The bacteria were grown in the L. B. media and cells were harvested when they reached the active phase, after seven hours. This was done by centrifuging at 10,000 g for 10 min. at 4^0 C. The cell mass were then washed with sterile cold water, five times, to remove media components. The cells were then weighed and used. Reactions were carried out in 250 ml Erlenmeyer flasks. The Lab-Line orbital shaker equipped with thermal control and air quality were used. The sample was taken out aseptically. Bacterial cells were removed from the sample by centrifuging into eppendrof and sample was analyzed. At all the steps, conditions were kept sterile.

2.3.6. Urea Determination :

Urea concentrations were determined using quantitative colorimetric blood urea nitrogen in serum / plasma determination kit from Sigma Chemical Co. USA (Koritz and Cohen, 1954).

2.3.7. Ammonia Determination:

Ammonia was analyzed using Fluorescent Light Scattering Multistat III microcentrifugal analyzer. This was based on reductive amination of 2-oxoglutarate, using gluterate dehydrogenase (GLDH), and reduced nicotinamide adenine dinucleotide (NADH). The decrease in absorbance at 340 nm due to oxidation of NADP, is proportional to the ammonia concentration.

2.4 RESULTS AND DISCUSSION

Experiments were designed to evaluate the possible use of microencapsulated *E. coli* DH 5 cells, having urea inducible *K. aerogenes* gene, for the removal of urea. The reaction media has similar composition as body fluids. The urea concentrations used were those encountered in kidney failure cases. The urea depletion kinetics of free bacteria in the reaction media were determined. Results are shown in Fig. 2.1. Results demonstrate that the bacteria are able to reduce urea concentration from 100.00 ± 1.00 mg / dl to 1.30 ± 0.18 mg / dl in 30 minutes.

The next study is to investigate whether during urea utilization bacteria produce ammonia. Also, if bacteria is capable of removing ammonia present in the original reaction solution. Figure 2.2 shows the obtained results. It is observed that bacteria do not produce ammonia, rather they are using ammonia. The original ammonia concentration in the medium, was 758.00 ± 70.00 μM. Most of this initial ammonia (758.00 ± 70.00 μM) was removed in 20 minutes when the concentration was only 90.42 ± 38.05 μM.

The next experiment is to study the urea depletion kinetics of alginate encapsulated bacteria. Results are shown in Fig. 2.3. Both free and encapsulated bacteria reduced the urea concentration. The encapsulated bacteria are able to lower urea from its original concentration 100.00 ±1.00 mg / dl to a concentration of 1.55 ± 0.13 mg / dl in 30 minutes. There are no major differences between urea depletion profiles of free and encapsulated bacteria, but free bacteria utilize urea at a slightly faster rate.

The encapsulation process does not affect urea depletion efficiency of the bacteria. Using a single pool model, we calculated the removal of urea by encapsulated bacteria for the total body water of 40 litre. It is found that 40 g of microencapsulated bacterial cells can reduce the urea in the 40 liters (100.00 mg / dl) to 6.00 mg / dl in 30 minutes. The encapsulated bacteria can remove about 98.50 % of urea from the total body fluid in 30 minutes without producing ammonia. This efficiency is sufficient to remove the desired amount of urea in kidney failure. This is 10 times the efficiency of oxystarch gel. Oxystarch adsorbs 103.00 mg of urea / g of oxystarch, at pH 7.4 and a urea concentration of 0.02 M (Espistino and Girdano, 1980). To remove 40 g of urea from the total body water, therefore, 388.34 gram of oxystarch is needed, when the urea concentration is 100.00 mg / dl. The microencapsulated bacterial system is about 30 time more efficient than the microencapsulated zirconium-phosphate-urease. This system removes 1.60 milligram of nitrogen or 33.00 mg urea / g of microcapsules. Thus, 1212.12 g microcapsules containing zirconium phosphate-urease would be required to remove 40 g of urea from the total body water (Kjellstrand et al., 1981). Thus, the efficiency of urea removal by microencapsulated engineered bacteria is 10 to 30 fold higher than previous urea removal systems. We are using a novel method to microencapsulate the bacteria (Wong and Chang, 1991) to prevent any leakage of bacterial cells.

2.5 CONCLUSIONS

In these studies, a novel method for urea and ammonia removal by microencapsulated genetically engineered *E. coli* DH 5 cells has been investigated. The alginate microencapsulated genetically engineered *E. coli* DH5 cells were found to deplete urea efficiently. The urea depletion efficiency was not affected due to microencapsulation of bacteria. The encapsulated bacteria was able to reduce the 100.00 ±1.00 mg / dl of urea to 1.50 ± 0.0013 mg / dl in 30 minutes when 100 milligram of cells were used. In similar conditions bacteria were able to lower ammonia from 758.00 ± 70.00 μM to 90.42 ± 38.05 μM in 20 minutes. The bacteria do not produce ammonia during urea utilization. Extrapolation of the depletion kinetics reveals that bacteria are efficient enough to remove urea during kidney failure. These findings are very encouraging and suggest its further development for urea and ammonia removal in kidney or liver failure.

2.6 ACKNOWLEDGMENTS

The grant support of the Medical Research Council of Canada and Quebec Ministry of Higher Education Science and Technology "Center for Excellence in Biotechnology Virage" Awards to T. M. S. C. is acknowledged. S. P. is a National Overseas Scholar from Government of India. The authors appreciate the technical assistance of Mr. Colin Lister. The generous gift of the organism used in this study by Prof. R. P. Hausinger is gratefully acknowledged.

2.7 REFERENCES

Chang, T.M.S. (1962). Semipermeable microcapsules. *Science*, 146, 524 p.p..

Chang, T.M.S. (1980). *Trans. Am. Soc. Artifi. Internal Organs,* 12, 13 p.p..

Chang, T.M.S. (1988). *Methods in Enzymology*, 137, 444-457 p.p..

Chang, T.M.S. (1991). *Artificial Cells" in Encyclopaedia of Human Biology* (Ronalo Dulbecco, ed.), Vol. 1, 377-383 p.p., Academic Press, San Diego, C.A.

Chang, T.M.S. (1992). *Biomat. Artfi. Cells & Immob. Biotechnol.*, 20(5), 1121-1144 p.p..

Esposito, R. and Giordano, C. (1980) : *Sorbents and their clinical applications (Giordano, C., Ed.)* ; 130-153 p.p.. Academic Press, New York.

Gu, K . F. and Chang, T.M.S. (1990). *Appl. Biochem. and Biotech.,* 26, 263-269 p.p..

Kjellstrand, C., Borges, H., Pru, C., Gardner, D. and Fink, D.(1981). *Trans. Am. Soc. Artificial Internal Organs,* 27, 24-30 p.p..

Koritz, S. B. and Cohen, P. P. (1954). *J. Biol. Chem.*, 209, 145 p..

Maniatis, T., Fritsh, B. D., and Sambrook, J. (1994). *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory, New York.

Mobley, H. L. and Hausinger, R. P.(1989). *Microbiolo. Rev.*, 53(1), 85-108 p.p..

Mulrooney, S. B., Martha, J. L., Mobley, H. L. T., and Hausinger, R. P. (1988). *J. of Bacteriol.*, 170 (5), 2202-2207 p.p..

Mulrooney, S. B., Sturart, P., and Hausinger, R .P. (1989). *J. of Gen. Microbiol.*, 135, 1769-1776 p.p..

Setala, K., Heinonen, H., and Purola, S. (1973). *J. Int. Res. Comm. System. 73,* 35 p.p.

Stephen, E. W., Susan, K. W., Sheila, I. H., and Rechard, A. H. (1988). *J. Bacteriolo.*, 170, 1027-1033 p.p..

Wong, H. and Chang, T.M.S. (1991). Biomat. Artifi. Cells & Immob. Biotechnol., 19(4), 675-686 p.p.

Wong, H. and Chang, T.M.S. (1991). Biomat. Artfi. Cells & Immob. Biotechnol., 19(4), 687-697 p.p..

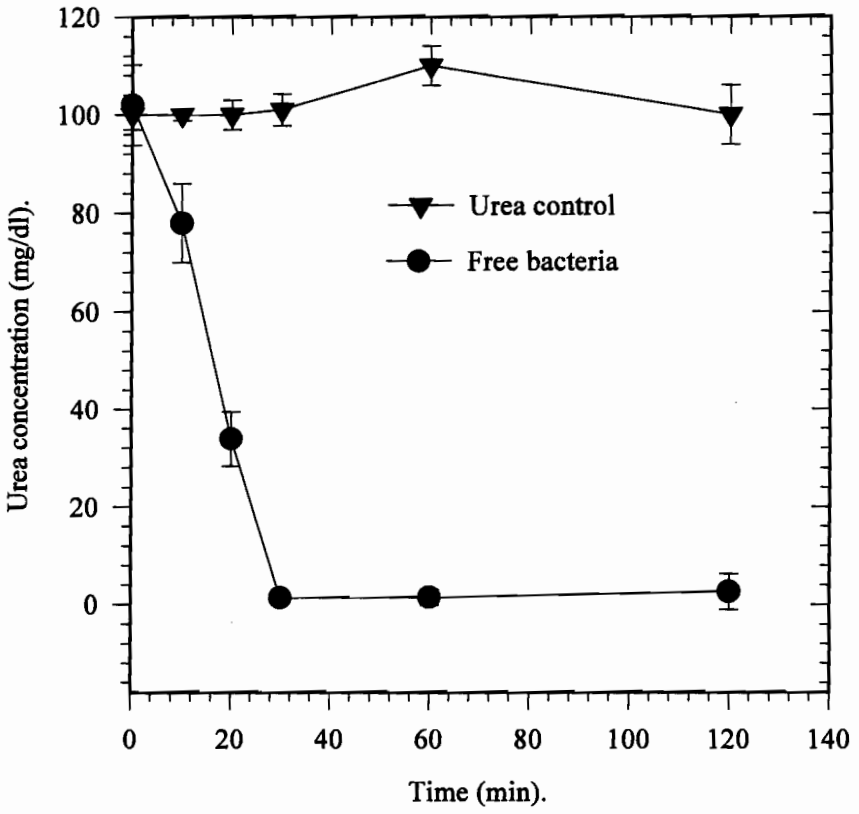


Figure 2.1 : Urea depletion profile of free bacteria. A quantity of 100 mg, 7 hr old active phase bacterial cells and 100 ml reaction media, pH 7, 30 ^{0} C and 100 rpm are the reaction conditions.

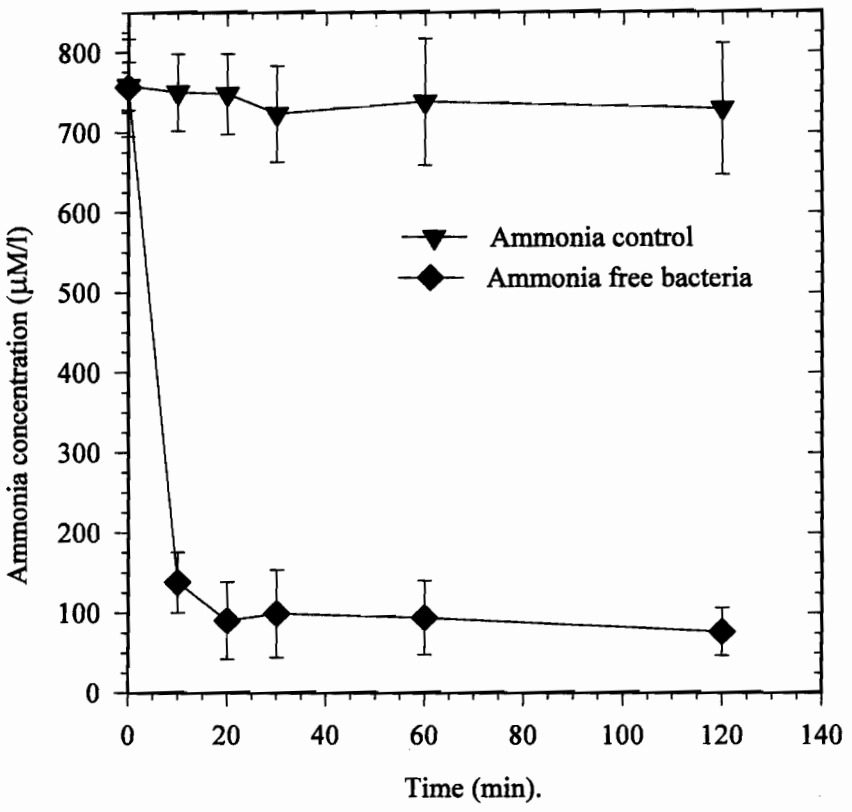


Figure 2.2 : Ammonia depletion profile during urea depletion by free bacteria. A quantity of 100 mg, 7 hr old active phase bacterial cells and 100 ml reaction media, pH 7, 30 ^{0} C and 100 rpm are the reaction conditions. The reaction media contains 100 mg/dl urea. The urea removal rate is similar to that shown in figure 2.1.

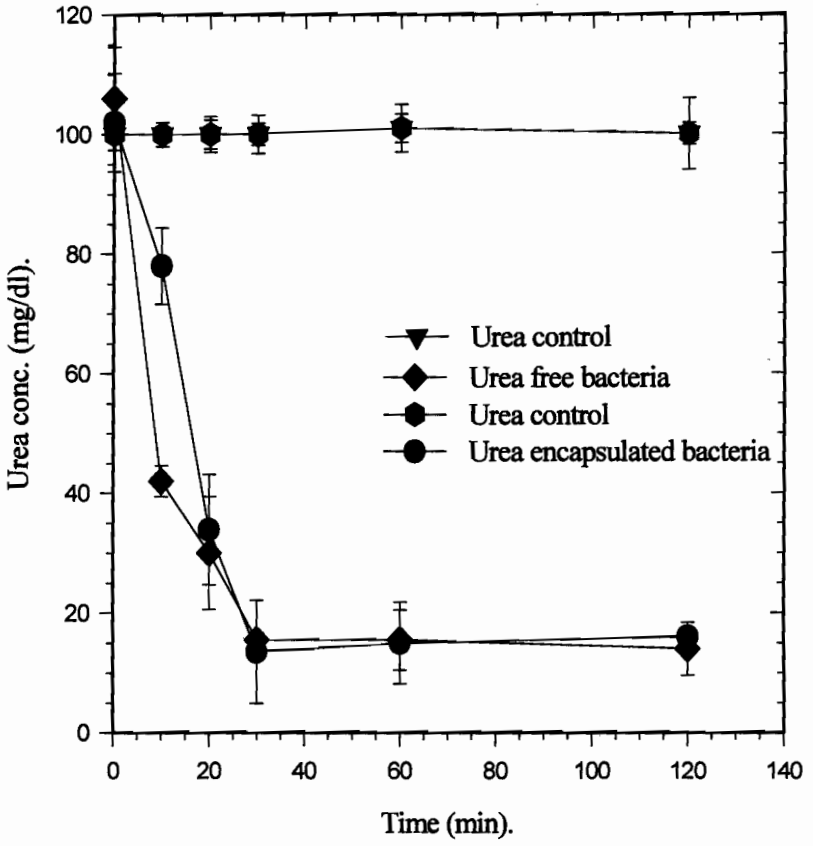


Figure 2.3: Urea depletion kinetics of free and encapsulated bacteria. A quantity of 100 mg, 7 hr old active phase bacterial cells and 100 ml reaction media, pH 7, 30 ^{0} C and 100 rpm are the reaction conditions.

APPENDIX 1.1

1.1 METHOD FOR UREA DETERMINATION

1.1.1 BACKGROUND AND PRINCIPLE OF THE TEST

The method used for urea measurement was based on the direct interaction of urea with diacetyle monooxime. This reaction was first described by Fearon (1939) and the results of his reaction was incorporated into a quantitative assay by Ormsby (1942) for the determination of urea in the blood and urine. Several modifications of the Ormosby's method have been proposed (Carolyn and Cracker, 1967; Wenk et al., 1973). The method used in this study is based on the modification by Carolyn and Crocker (Carolyn and Cracker, 1967) given for the determination of serum or plasma urea without deproteinization. The reaction involved in the process is as follows :

Urea + Diacetyle monooxamine ----------> Chromogen (Pink Colour) + Hydroxylamine.

Urea concentration is directly proportional to the intensity of the colour produced, which can be measured spectrophotometrically between 515-540 nm. This method is simple, rapid, highly sensitive, and is not influenced by the presence of ammonium ions (Wenk et al., 1973).

1.1.2 REAGENTS

a) BUN ACID REAGENT sigma catalogue # 535-3

Ferric chloride in phosphoric and sulphuric acids. Stored at room

temperature (18-26 ^{0} C).

b) BUN COLOUR REAGENT sigma catalogue # 535-5
Diacetyle monoxime, 0.18 % [w/v], and thiosemicarbazide. Reagent performance is not affected by crystal formation and is suitable for the use in absence of visible microbial growth.

c) UREA NITROGEN STANDARD SOLUTION sigma catalogue # 535-30
Urea at a urea nitrogen level of 150 mg/dl (53.5 mol/L)with benzoic acid as a preservative.

1.1.3 SPECIMEN COLLECTION AND STORAGE

a) Media : For the media urea removal studies, specimen were collected into the ependroff tubes and analysed.

b) Plasma: Blood is drawn into a tube containing anticoagulant heparin, mixed and centrifuged, at 10,000 g at 4 ^{0} C, for 10 min and plasma was separated and analysed. Plasma may be stored for several days refrigerated or several month frozen without any appreciable loss of urea (Wenk et al., 19737).

1.1.4 INSTRUMENTS AND MATERIALS USED

Perkin-Elmer Lambda 4 B UV/VIS Spectrophotometer, centrifuge, pipette, test tubes, spectrophotometer cuvette, boiling water bath, pipette and timer.

1.1.5 ASSAY PROCEDURE

a) Level 3 or more test tubes, BLANK, STANDARD, TEST 1, TEST 2, TEST 3. etc.

b) To each tube, add 3 ml of BUN acid reagent and 2 ml of BUN colour

reagent. Mix thoroughly.

c) To the standard, add 20 µl of urea nitrogen standard and to the test add 20 µl of media or plasma. To the blank add 20 ml of water. Mix tubes thoroughly.

d) Simultaneously, place all tubes in boiling water bath for exactly 10 minutes.

e) Quickly remove the tubes and place them on ice cold water for 5 minutes.

f) Read absorbance of STANDARD, TEST vs. BLANK as reference at the same wavelength used in preparing the calibration curve.

1.1.6 PREPARATION OF STANDARD CURVE

a) Level the 11 test tubes as BLANK, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10.

b) Prepare the urea nitrogen diluted standards using 150 mg/dl stock urea solution and water. The final concentration used for the standard curve preparation was as follows : BLANK (0), 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/dl.

c) Pipette 20 µl of each of the diluted standard urea solution to the test tubes.

d) Add 2 ml of BUN acid reagent and 3 ml of BUN colouring reagent to each test tubes containing 20 µl standard urea solution.

e) Simultaneously place all tubes in boiling water bath for 10 minutes.

f) Quickly remove tubes and place in ice cold water for 5 minutes.

g) Within 20 minutes, read absorbance of tubes 1-10 vs. Blank as reference at 540 nm. Record the absorbance (optical density).

h) Standard curve, absorbance vs. corresponding urea nitrogen concentration (Figure 2.4) was prepared using average of the O. D. for three batches of the BUN acid and BUN colouring solution after statistical analysis of the data with 99 % confidence interval (table) using the sigma plot.

1.1.7 RESULTS

An excellent standard curve is obtained (Figure 2.4).

1.1.8 NOTES

i) Freshly prepared urea standard solution is required.

ii) Urea solution may be stored at 4^0 C.

1.1.9 REFERENCES

Carolyn, L. and Cracker, M.T. (1967). Rapid determination of urea nitrogen in serum and plasma without deporoteinization. Am. J. of Med. Tech., 33 (5),361-365.

Fearon, W. R. (1939). The carbamido diacetyle reaction; A test for citrulline. Biochem. J., 33,902.

Hunninghake, D. and Grisolia, S. (1966). A sensitive and convenient micromethod for the estimation of urea citrulline and carbamyl derivatives. Anal. Biochem. 16,200-205.

Marsh, W. H. Fingerhut, B. and Miller, H. (1965). Automated and manual direct methods for the determination of blood urea. Clin. Chem., 11, 624-627.

Ormmsby, A. A. (1942). A direct colorimetric method for determination of urea in blood and urine. J. Biol. Chem., 146,595.

Wenk, E., Lustgranten, J. A. and Byrd, C. (1973). An evaluation of "kits" for determining urea nitrogen. Am. J. Clin. Patho., 59,542.

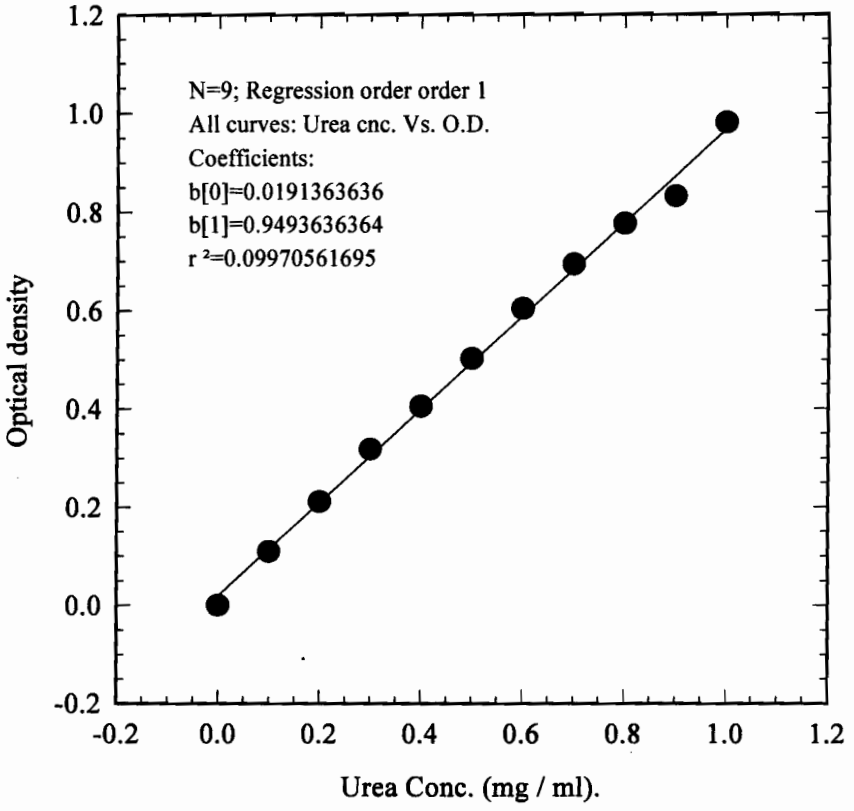


Figure 2.4: Standard curve for urea determination.

APPENDIX 1.2

1.2 MEHOD FOR AMMONIA DETERMINATION

The system used was Instrumentation Laboratories, Multistat III plus (Spokane, Wash.), microcentrifuge analyzer (MCA) in the absorbance mode (Sigma procedure, catalogue 170-UV). The multistat III plus system consisted of the loader, which dispensed sample and reagents into a maximum of 20 cuvettes arranged readily in a plastic rotor, and analyzer which performed tests on the filled cuvettes using spectrophotometry and centrifugation. There are three main devices involved in the analytical process on the multistat III plus system.

a) **Rotors :** The rotor is a precision device whose task is to receive precisely measured amounts of reagent and sample in separate cuvette compartments, to permit the mixing of the reagents and specimen at a specific rotational speed. It also permits various optical readings to be taken.

b) **Loaders :** The loader is a microprocessor-controlled device whose task is to dispense precisely measured amounts of diluent, reagents and sample in each cuvette of the rotor. Loading parameters for each test reside in the loader memory and may be reviewed and edited as required. A data cable transmits data from rotors to the analyzer.

c) **Analyzer :** The analyzer performs the selected analysis and prints out the results in appropriate units. Analytical parameters are loaded into computer memory from the multiprogram cassette tape, which also contains special programs for statistical analysis and data retrieval. After being prepared by the loader, the rotor is placed in the analyzer. The analyzer automatically controls the centrifugation, temperature, emission wavelength, readings, statistical analysis, calculations, and data print out.

1.2.1 BACKGROUND AND PRINCIPLE OF THE TEST :

Circulating ammonia levels reflect urea cycle activity as it relates to glucose protein, and nucleic acid metabolism, particularly in the liver. Accordingly, plasma ammonia concentrations serve as indicators of liver damage (e.g., Reye's syndrome) and possible impending hepatic coma (Ratliff, and Hall, 1979).

This method for ammonia determination in media or plasma is based on reactive amination of 2-oxoglutarate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH) as follows (Dewan, 1938).

$$\text{Oxoglutarete} + NH_3 + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}$$

The decrease in absorbance at 340 nm [A_{340}], due to oxidation of NADH, is proportional to the ammonia concentration.

1.2.2 REAGENTS

1.2.2.1 Ammonia control solution

Freshly prepared ammonium sulphate solution, ammonia 5 mg/ml or 294 μ mol /L ; or 2 mg/ml or 118 μ mol/L were used. Solution was stored at 2-6 ^{0} C.

1.2.2.2 Ammonia assay solution

Ammonia reagent solution was prepared by reconstituting ammonia reagent vial from sigma (catalogue no. 170-10, 10-ml size) with 10 ml deionized water. After reconstitution, reagent containing 2-oxoglutarate, 2 mmol/L, NADH, 0.12 mmol/L, with buffer and stabilisers. Ammonia assay solution is stable for at least 8 hours at room temperature (18-26 ^{0} C)

and 2 days when refrigerated (2-6 ^{0} C). Freezing extends stability to 1 week.

1.2.2.3 L-GLDH Solution

L-Glutamate dehydrogenase (bovine liver), 1200 units/ml, in 50 % glycerol and phosphate buffer, pH 7.4. Stored at 2-6 ^{0} C.

1.2.2.4 Working ammonia reagent

To 5.0 µl of ammonia reagent prepared above, 75 µl of GLDH was added and swirl gently to mix.

1.2.3 SPECIMEN COLLECTION AND STORAGE

Sample was collected from the reaction media and analysed, when required centrifugation was performed to remove bacteria. For the plasma ammonia removal studies, blood is collected from the tail of the rats using the verified ammonia free heparin as the anticoagulant. Heparin is the preferred anticoagulant over other anticoagulant such as sodium citrate, potassium oxalate or sodium fluoride, because it reduces red cells ammonia production (Diamond, 1955). Stability of the ammonia into the heparinized plasma is said to be stable up to 3 hours stored in an ice bath (Conn, 1965). Stability of the ammonia in the heparinized blood plasma can be extended up to 3 days if the specimens are kept frozen in liquid nitrogen or on dry ice (Conn, 1965).

1.2.4 ASSAY PROCEDURE

a) All the necessary reagents were prepared as quickly after the specimen has been collected as possible. And as soon as the specimen was separated, they were kept on ice bath until everything else was prepared.

b) Load the multistat rotor with standards, controls and specimen (media or plasma in-vitro and in-vivo studies) using the loader settings for MPL type of loader. The ring was always loaded in the following sequence :

1. Water
2. 200 μ mol /L ammonia standard
3. 300 μ mol /L ammonia standard
4. 400 μ mol l/L ammonia standard
5. 500 μ mol /L ammonia standard
6. 600 μ mol /L ammonia standard
7. 700μ mol l/L ammonia standard
8. Specimen solution.....

c) Reagent loading set-up

Reagent position 1 : working ammonia reagent + GLDH

Reagent position 2 : Ammonia reagent (no GLDH).

d) Loader setting for MPL type of rotors

Programme code #19

Volume sample 20 μl

Volume diluent 5 μl

Volume second reagent (regent at position second) 40 μl

Volume diluent 2 μl

Volume first regent (reagent at position first) 100 μl

First sample position (cuvette 2)

Last sample position

Reference cuvette (water, cuvette 1)

e) Once the instrument has been set up and rotors loaded, place the filled rotors into the analyzer. Answer the questions prompted on the analyzer and start the assay by pressing "enter". The analyzer mixed the sample with the reagent and incubated it for 5 min at 37 ^{0} C and read the

absorbance at 340 nm.

f) For each batch of the assay operation was performed using separate standard ammonia solution in the rotors having ammonia concentration ranging from 200 -700 μ mol.

d) The specimen ammonia concentration will come out ready to report.

1.2.5 RESULTS

Figure 2.5 summarises the standard curve for ammonia measurement.

1.2.6 NOTES

i) Plasma sample should be centrifuged before freezing.

ii.) A separate standard ammonia solution should be used for each set of experimental ammonia determination experiments.

1.2.7 REFERENCES

Conn, H. O. (1965). Studies on origin and significance of the blood ammonia. 1. Effect of various anticoagulants on blood ammonia determination. Vol.5; In: S. Meites (ed.). Academic press, N.Y.

Dewan, J. G. (1938). L (+)-glutamate dehydrogenase of animal tissues. Biochem J., 32,1378.

Diamond, E. G. (1955). Inhibitory effect of heparin upon adenylic deaminase. J. Lab. Clin. Med, 46, 807.

Ratliff, C. R. and Hall F. F. (1979). Blood ammonia returns to the laboratory. Laboratory management, Aug. page 16.

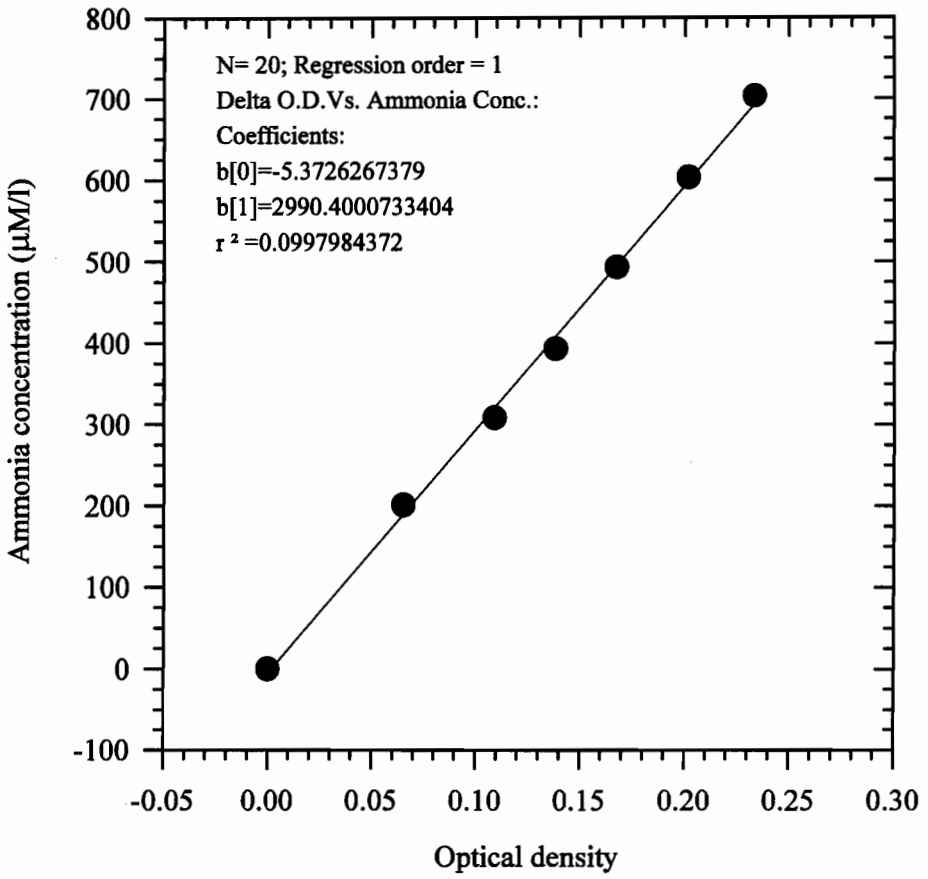


Figure 2.5 : Standard curve for ammonia determination.

CHAPTER 3

Preparation and In-Vitro analysis of Microencapsulated Genetically Engineered *E. coli* DH5 Cells for Urea and Ammonia Removal

S. Prakash and T. M. S. Chang

Biotechnology and Bioengineering;
Vol. 46(6), page 621-626, 1995.

3.1 ABSTRACT

This article describes a novel method of urea and ammonia removal using microencapsulated genetically engineered *Escherichia coli* DH5 cells. Optimization of bacterial cell encapsulation was carried out. The optimal method consists of alginate 2.00 % (w/v) at a flow rate of 0.0724 ml/min and a coaxial air flow rate of 2.00 L/min. This produces spherical, alginate-poly-L-lysine-alginate (APA) microcapsules of an average 500 ± 45 μm diameter. Increasing the concentration of alginate from 1.00 % to 1.75 % improves the quality of the microcapsules, while cell viability remains unaffected. The APA microcapsules are mechanically stable up to 210 rpm agitation with no bacterial cell leakage. The in-vitro performance of urea and ammonia removal by encapsulated bacteria is assessed. One hundred milligrams of bacterial cells in APA microcapsules, in their log phase state of growth, can lower 87.89 ± 2.25 % of the plasma urea within 20 minutes and 99.99 % in 30 minutes. The same amount of encapsulated bacteria can also lower ammonia from 975.14 ± 70.15 μM/L to 81.151 ± 7.37 μM/L in 30 minutes. There are no significance differences in depletion profiles by free and encapsulated bacteria for urea and ammonia removal. This novel approach using microencapsulated genetically engineered *E. coli* cells is significantly more efficient than presently available methods of urea and ammonia removal. For instance, it is 30 times more efficient than the standard urease-ammonium adsorbent system.

Key words : microencapsulation. urea. ammonia. alginate-poly-L-lysine-alginate (APA). artificial cells. mechanical strength.

3.2 INTRODUCTION

Urea and ammonia removal are needed in kidney failure (Castelman and Mineey, 1974; Charles and Stacia, 1990; Cattaneo and Chang, 1991), liver failure (Castelman and Mineey, 1974; Chang, 1985, 1990; Charles and Stacia, 1990), environmental decontamination, and regeneration of water supply in space travel (Dayton, 1989). Standard dialysis are usually complex and expensive; thus, several alternatives have been considered (Chang, 1966; Walker et al., 1977; Friedman, 1978; Sparks, 1979; Chang, 1980; Kjellstrand et al., 1981; Chang, 1988; Drukker et al., 1983; Gu and Chang, 1988; Kolff, 1990; Cattaneo and Chang, 1991). The use of a microencapsulated reactant (Spark, 1979), enzyme urease to convert urea into ammonia, and its subsequent removal by chemical adsorbents was proposed and demonstrated (Chang, 1966, 1980, 1988; Cattaneo and Chang, 1991). This was based on the concept and principle of artificial cells (Chang, 1965). However, ammonia adsorbent available at present does not have sufficient adsorbent capacity (Sparks et al., 1972; Gu and Chang, 1988). Other approaches include the use of oxystarch (Friedman, 1978) or urea utilizing soil bacteria (Setala et al., 1927, Setala et al., 1973). The use of oxystarch was encouraging, but is associated with high dose requirements and low in-vivo urea removal efficiency, particularly at a neutral pH (Espostino and Carmelo, 1980). Similarly, lyophilized soil bacterial cells were not sufficiently effective. In another approach, an encapsulated multienzyme system that converts urea and ammonia into essential amino acids was investigated (Chang, 1980; Chang, 1985; Gu and Chang, 1988,1991). The encapsulated multienzyme system did not have a sufficiently high rate of urea and ammonia conversion.

The methods for microencapsulation of biologically active materials (Chang, 1965) are well established and offer a range of biomedical applications (Sparks, 1979; Kjellstrand et al., 1981; Chang, 1985; Goosen et al., 1985; Chang, 1988, 1993, 1994; Prakash and Chang, 1993). The use of a system that utilizes urea or ammonia directly

may be the most appropriate approach in removing urea and ammonia. We have published a preliminary report that microencapsulated genetically engineered *Escherichia coli* cells, containing the *Klebsiella aerogens* urease gene, can efficiently remove urea from the reaction media without producing ammonia (Prakash and Chang, 1993). Since then, we have carried out more detailed studies on the optimization of process parameters for bacterial cell encapsulation in APA membrane, urea and ammonia removal profiles of genetically engineered APA-encapsulated bacteria, and efficacy of urea and ammonia removal in-vitro.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals:

Alginic acid (low viscosity, Lot 611994) and poly-L-lysine (MW viscosity 16,100, Lot 11H5516) were purchased from Kelco and Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively. All other chemicals were of analytical reagent grade.

3.3.2 Microorganism and Culture Conditions:

Genetically engineered bacteria *Escherichia coli* DH5, containing the urease gene from *Klebsiella aerogens*, was a generous gift from Prof. R. P. Haussinger (Mobely and Haussinger, 1989; Mulrooney et al., 1989). Luria-Bertani (LB) growth medium was used for primary cell cultivation. The composition of LB medium was of 10.00 g/L bactotryptone (Difco), 5.00 g/L bacto yeast extract (Difco), and 10.00 g/L sodium chloride (Sigma). The pH was adjusted to 7.5 by adding about 1.00 ml of 1.00 N NaOH. Media were then sterilized in Castle Labclaves for 30 minutes at 250 ^{0} F. Incubation was carried out in 5.00 ml LB in 16.00 ml culture tubes at 37 ^{0} C in an orbital shaker at 120

rpm. For the large-scale production of biomass, for microencapsulation purpose, 250 ml Erlenmeyer flask containing 100 ml LB medium was used.

3.3.3 Microencapsulation :

Microcapsules containing bacterium *E. coli* were prepared by the method of Lim and Sun (Lim and Sun, 1980) with a modification of Wong and Chang (Wong and Chang, 1991) using the apparatus set-up assembly shown in Figure 3.1 A. Bacterial cells were suspended in an autoclaved sodium alginate in 0.9 % sodium chloride solution. The viscous alginate-bacterial suspension was pressed through a 23 gauge needle using a syringe pump (Compact Infusion Pump Model 975, Harvard App. Co. MA). Compressed air through a 16 gauge needle was used to shear the droplets coming out of the tip of the 23 gauge needle. The two needles in combination make up the droplet needle assembly in Figure 3.1 B. The droplets were allowed to gel for 15 minutes in a gently stirred ice-cold solution of calcium chloride (1.4 %). After gelation in the calcium chloride, alginate gel beads were coated with polylysine (0.05 % in HEPES buffer saline, pH 7.20) for 10 minutes. The beads were then washed with HEPES and coated with an alginate solution (0.1 %) for 4.00 minutes. The alginate-poly-L-lysine-alginate capsules were then washed in a 3.00 % citrate bath (3.00 % in 1:1 HEPES-buffer saline, pH 7.20) to liquify the gel in the microcapsules. The microcapsules formed were stored at $4\ ^{0}$ C and used for the experiments.

3.3.4 Reaction Media for Urea and Ammonia Removal Experiments:

Reaction media in all experiments consisted of 1.00 g/L glucose, 20.00 mg/L magnesium sulphate, 30.00 mg/L dipotassium monohydrogen phosphate, and 0.07 mg/L vitamin B_{12}. As required, filtered sterilized urea was added in the reaction media to make the urea concentration 100 mg/dl. Plasma from a bovine source was used for the plasma urea and ammonia removal experiments and filter-sterilized urea was added to the

plasma. This was done to mimic the uremic patient's plasma characteristics in vitro (Castelman, 1974).

3.3.5 Experimental Procedure:

The bacteria were grown in L B medium. Log phase bacterial cells were harvested by centrifuging at 10,000 g for 20 min. at 4 ^{0} C. The cell mass was then washed five times with sterile cold water to remove media components. Cells were then weighed and used for the urea and ammonia removal studies by free bacteria. For the microencapsulated urea and ammonia removal studies the equivalent masses of the cells were microencapsulated and used. In all experiments, reactions were performed in 250-ml Erlenmeyer flasks at 30 ^{0} C and 100 rpm, unless otherwise mentioned. The Lab-Line orbital environ-shaker equipped with thermal control and air quality were used for this purpose. Sampling was carried out aseptically at designated times. Bacterial cells, in the free bacteria removal studies, were removed from the sample by centrifugation at 15,000 rpm and the supernatant analyzed. For the plasma urea and ammonia removal studies we used bovine plasma with filter-sterilized urea added to a final urea level comparable with that of kidney failure patients (Castelman, 1947; Epastino and Carmelo, 1980). In all experiments, the ratio of the reactor volume to the amount of microencapsulated bacteria used was held constant.

3.3.6 Urea and Ammonia Determination:

Urea concentrations were determined based on quantitative measurements of blood using the BUN kits purchased from Sigma Chemical Co. U.S.A. (Symmour and Philips, 1954). Ammonia was analyzed using a fluorescent light scattering Multistat III microcentrifugal analyzer. This was based on the reductive amination of 2-oxoglutarate, using glutarate dehydrogenase (GLDH), and reduced nicotinamide adenine dinucleotide

(NADH). The decrease in absorbance at 340 nm due to the oxidation of NADH is proportional to ammonia concentration.

3.4 RESULTS AND DISCUSSION

Alginate based membranes such as alginate, alginate-polyl-L-lysine (AP), alginate-PLL-PEI, and alginate-poly-L-lysine-alginate (APA) are the most commonly used membranes for microencapsulation of cells. (Chang, 1980; Lim and Sun, 1980; Kjellstrand et al., 1981; Goosen et al., 1985; Chang, 1988; Martison et al.,1989; Chang, 1990; Chang, 1993; Pat et al., 1993; Chang, 1994). Alginate beads, spherical in shape and small in diameter, with uniform size distribution, are necessary to obtain strong microcapsules with appropriate characteristics. The effect of alginate concentration on microcapsule morphology, diameter, and cell viability are shown in Table 3.I. Results show that alginate concentration in the tested range, 1.00 to 2.25 % (w/v), does not affect the bacterial cell viability. Also, cell growth inside the microcapsules is independent of alginate concentration in the tested range. Furthermore, quality of microcapsules improves with increasing alginate concentration from 1.00 % to 1.75 % (w/v). However, there was no significant effect of alginate concentration on size of the microcapsules. But the percent fraction of perfectly formed microcapsules increased with increasing alginate concentration from 1.00 % (w/v) to 1.75 (w/v). Overall, using 2.00 % (w/v) alginate resulted in a perfectly spherical shape and sturdy microcapsules with a maximum number of encapsulated bacterial cells. The presence of nonspherically shaped microcapsules may be eliminated by adjusting the height of the needle assembly or alginate concentration.

The effect of air flow rate on size of microcapsules is shown in Figure 3.2. Size of the microcapsules decreases with increasing air flow rate. At an air flow rate of 2.00 L/min, the microcapsules had an average diameter of 500 ± 45 μm. At air flow rates

above 3.00 L/min, microcapsules were found to be irregular in shape, possibly due to fragmentation created by higher shear forces at the tip of the needle assembly. Effect of flow rate of the alginate-bacterial suspension through the syringe pump and the size of the microcapsules was also determined. Figure 3.3 shows that an increase in liquid flow from 0.00264 to 0.0369 ml/min resulted in increase in microcapsule diameter. Phase contrast microscopy revealed that only microcapsules made with liquid flow rates of 0.00724 to 0.278 ml/min are uniformly spherical in shape. These results indicate that alginate concentration, air flow rate, and liquid flow rate are critical for obtaining microcapsules of desired characteristics. Thus, using 2.00 % (w/v) alginate, 0.0724 ml/min liquid flow rate, and 2.00 L/min air flow rate lead to superior microcapsules for subsequent experiments.

The mechanical strength of the microcapsules as a function of cell leakage is then determined. For this, alginate beads and APA microcapsules, 100 microcapsules of each type, were thoroughly washed and agitated in L B medium in 250-ml flasks at $30\ ^{0}$ C on an orbital shaker for 7 hours. Results (Fig. 3.4) show that alginate microbeads are stable up to an agitation of 190 rpm, compared with 210 rpm for APA microcapsules, in terms of cell leakage.

In the present studies, we compared the in-vitro effectiveness of urea removal from aqueous solution and from plasma to study the use of the same encapsulated, genetically engineered bacteria for removal of urea and ammonia in biotechnology, chemical engineering, and biomedical applications. The urea depletion profiles of encapsulated bacteria were determined and results are shown in Figure 3.5. Log phase microencapsulated bacteria lowered 87.89 ± 2.25 % of plasma urea within 20 minutes and 99.99 % of urea in 30 minutes. Results, when compared, show (Fig. 3.6) that encapsulated bacteria are slightly more effective in removing urea from plasma than from modified reaction media.

The next study was to investigate whether, during urea utilization, ammonia is produced. A study was also carried out to see if microencapsulated, genetically engineered bacteria are capable of removing high plasma ammonia comparable with that in liver failure. Results show that the bacteria do not produce ammonia during urea utilization. Furthermore, Figure 3.7 shows that encapsulated bacteria decreases plasma ammonia concentration from 975 ± 70.15 μM to 81.15 ± 7.37 μM in 30 min. When compared, it was found that ammonia removal efficiency of encapsulated bacteria in plasma is not significantly different than that in aqueous media (Fig. 3.8). This efficiency of ammonia removal is better than currently used methods for ammonia removal (Friedman, 1978).

Figure 3.9 shows that one can use encapsulated bacteria up to three cycles. APA-encapsulated bacteria plasma urea removal rate is greater in the second and third cycles then in the first. This is probably due to an increase in total biomass inside the microcapsules with time. There is no leakage of encapsulated bacteria in the first, second, and third cycles. The cumulative urea removal capacity of encapsulated bacteria is determined for the three cycles and the results are shown in Figure 3.10. At present these encapsulated bacteria can only be used for three cycles. Bacteria leakage occurs when encapsulated bacteria are used for a fourth cycle. It is important to use this approach separately for more cycles. The two step procedure for microencapsulation (Wong and Chang, 1991) may allow encapsulated bacteria to be used for even more cycles.

Using a single pool model, the percent of plasma urea removal efficiency by the encapsulated bacteria from a 40-L body fluid compartment was calculated. The urea removal capacity of encapsulated bacteria was calculated and compared with other standard bioreactants used for urea and ammonia removal (Fig. 3.11 left). A quantity of 40.00 ± 8.60 g of APA-encapsulated bacteria can remove 87.89 ± 2.25 % of the plasma urea within 20 minutes and 99.99 % of the urea in 30 minutes from the 40-L fluid compartment (Fig 3.11 right). Based on this calculation, encapsulated bacteria are ten fold

more efficient than oxystarch. One gram of oxystarch adsorbed 103.00 mg of urea at pH 7.4 at a urea concentration of 0.02 M (Espostio and Carmelo, 1980). Thus, to remove 40 g of urea from 40 L of fluid (100 mg / dl urea) 388.34 g of oxystarch is needed. Microencapsulated, genetically engineered bacteria are also 30 times more efficient compared with microencapsulated enzyme urease-zirconium-phosphate. The encapsulated urease-zirconium-phosphate system removes 1.60 mg of urea nitrogen or 33.00 mg urea / g of microcapsules (Espostio and Carmelo, 1980). Thus, 1212.12 g of microcapsule containing urease-zirconium-phosphate would be required to remove 40 g of urea from the total body water. Overall, urea removal efficiency of microencapsulated, genetically engineered bacteria is 10 to 30 times higher than that of existent urea removal systems (Fig. 3.11).

3.5 CONCLUSIONS

This study describes a novel method for plasma urea and ammonia removal by microencapsulated genetically engineered bacterial cells. The APA-encapsulated genetically engineered *E. coli* cells efficiently deplete urea. Urea depletion was unaffected by the microencapsulation process. Two percent (w/v) alginate concentration, 0.0724 ml/min alginate-bacterial cell suspension flow rate, and 2.00 L/min. air flow rate produces spherical and sturdy APA microcapsules of 500 ± 45 μm in diameter. They are mechanically stable up to 210-rpm agitation; that is, no cell leakage occurs. The alginate concentration in the tested range in this study did not affect cell viability. Encapsulated bacteria was able to reduce 87.89 ± 2.25 % of the total plasma urea within 20 minutes and 99.99 % of urea in 30 minutes. Under similar conditions, the same bacteria can lower plasma ammonia from 975.14 ± 70.15 μM to 81.15 ± 7.37 μM in 30 minutes. Bacterial cells do not produce ammonia and use urea for its metabolic nitrogen requirement. Our studies show that microcapsules are reusable for three cycles before cell leakage occurs.

Results indicate that this biotechnological approach is 10 to 30 times more efficient for removing urea and ammonia than the standard approaches.

3.6 ACKNOWLEDGEMENTS

The generous gift of the organism used in this study by Prof. R P. Haussinger is gratefully acknowledged. The authors gratefully acknowledge the research support of the Medical Research Council of Canada. The support of the Quebec Ministry of Higher Education Science and Technology "Center for Excellence in Biotechnology Virage" to T. M. S. C. and National Overseas Scholarship from the government of India to S. P. is thankfully acknowledged. The technical assistance of Mr. Colin Lister is also acknowledged.

3.7 REFERENCES

Castelman, B. and McNeely, B. U. (1974). Case records of the Massachusetts General Hospital. N. Engl. J. of Med. 290: 39-49.

Cattaneo, M., Chang, T.M.S. (1991). The potential of microencapsulated urease-zeolite oral sorbent for the removal of urea in uraemia. J. Am. Soc. Artif. Intern. Org. 37: 80-87.

Chang, T. M. S. (1964). Semipermeable microcapsules. Science. 146: 524-525.

Chang, T. M. S. (1966). Semipermeable aqueous microcapsule (artificial cells) : With emphasis on experiments in extracorporeal shunt systems Trans. Am. Soci. Artifi. Intern. Org. 12: 13-19.

Chang, T. M. .S. (1980). New approaches using immobilized enzymes for the removal of urea and ammonia. Enzyme Eng. 5: 225-229.

Chang, T. M. S. (1985). Biotechnology of artificial cells including application to artificial organs, p.p. 53-73. In: M. Moo Young (ed.), Comprehensive biotechnology: The principle applications & regulation of biotechnology in industry, agriculture, and medicine. Pergamon press, New York.

Chang, T. M. S. (1988). Methods in the medical application of immobilized proteins, enzymes and cells. Methods in Enzymology. 137: 444-457.

Chang, T. M. S. (1990). Biotechnological and medical application of immobilization of hepatocytes, microorganisms and enzyme systems by microencapsulation in artificial cells. Enz. Eng. 10: 331-339.

Chang, T. M .S. (1993). Living cells and microorganisms immobilized by microencapsulation inside artificial cells, pp. 183-194. In: M. F. Goosen (ed.), Fundamentals of animal cell encapsulation and immobilization. CRC press, Boca Raton, FL.

Chang, T. M. S. (1994). Artificial cell including blood substitute and biomicroencapsulation. Artif. Cells Blood Subst. and Immo. Biotechnnol. 22: vii-iv.

Charles, S., David, M., Stacia, M. (1990). pp. 526-527. In : A Deborah (ed.), Human physiology: foundations and frontiers. Times mirror college publi., St. Louis.

Dayton, L. (1989). Cells by design. New Scientist. 3 June: 42-43.

Drukker, W., Parsons, F. M., and Mahar, J. F. (1983). Replacement of renal functions by Dialysis. Martinus Nijhoff, Boston.

Esposito, R., Carmelo, G. (1980). Polyhalides. pp.133-153. In: G. Caramelo (ed.), Sorbents and their clinical applications. Academic press, London.

Friedman, E. A. (1978). pp. 521-528. In: E. A. Friedman (ed.), Strategy in renal failure. Willey, New York.

Goosen, M. F. A., O'Shea, G. M., Gharapetiean, H. M., and Sun, A. (1985). Optimization of microencapsulation parameters: semipermeable microcapsules as a bioartificial pancreas. Biotechnol. Bioeng. 27: 146-150.

Gu, K. F., and Chang, T. M. S. (1988). Conversion of alpha-ketoglutarate into L-glutamic acid with urea as ammonia source using multienzyme system and NAD immobilized by microencapsulation with artificial cell in bioreactor. Biotechnol. Bioeng. 32: 363-368.

Gu, K. F. and Chang, T.M.S. (1991). Conversion of urea or ammonia in to essential amino acids, L-leucine, L-valine and L-isoleucine using artificial cells containing an immobilized multienzyme system and dextran-NAD. Yeast alcohol dehydrogenase for enzyme recycling. J. Biotech. Applied Biochem. 12: 227-236.

Kjellstrand, G., Borger, H. Pru, C. G. and Fink, D. (1981). Trans. Am. Soc. of Artif. Intern. Org. 27: 24-30.

Kolff, W. J. (1990). Artificial kidney and artificial heart: Future considerations. Int. J. Artif. Org. 13: 404-406.

Kong, H. L., Pat, M. L., and Yew, S. S. (1993). Immobilization of aminoacylase by encapsulation in poly-L-lysine stabilized calcium alginate beads. J. Chem. Tech. Biotech. 37: 27-32.

Kwok, W. Y., Kiparissides, C., Yuet, P., Harris, T. J., and Goosen, M. F. (1991). Mathematical modeling of protein diffusion in microcapsules: A comparison with experimental results. Can. J. Chem. Eng. 69: 360-370.

Lim, F., and Sun, A. M. (1980). Microencapsulated islets as bioartificial pancreas. Science 210: 908-910.

Martinson, A., Skjak-Braek, G., and Smidsren, O. (1989). Alginate as immobilized material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol. Bioeng. 33: 79-89.

Mattiasson, B. (1983). pp. 3-35 In: B. Mattiasson (ed.), Immobilized cells and organelles., CRC Press, Boca Rotan, FL..

Mobley, H. L. and Haussinger, R. P. (1989). Microbial ureases: Significance, regulation, and molecular characterization. Microbiol. Rev. 53: 85-108.

Pat, M. L., Kong, H. L., and Yew, S. S. (1993). Covalent immobilization of aminoacylase to alginate for L-phenylalanine production. Chem. Tech. Biotechnol. 58: 65-70.

Prakash, S. and Chang, T. M .S. (1993). Microencapsulated genetically engineered *E coli* cells containing genes form *K. aerogens* for urea and ammonia removal. Biomat. Artifi. Cells Immob. Biotechnol. 19: 687-697.

Scoot, B., Mulrooney, H. Stuart, P., and Haussinger, R. P. (1989). Regulation of gene expression and cellular localization of cloned *K.aerogens* urease. J. Gen. Microbiol. 135: 1769-1776.

Setala, K., Heinonen, H., and Schreck-Purla (1973). Ingestion of lyophilized soil bacteria for alleviation of uraemic symptoms. J. Int. Res. Comm. Syst. 73.

Setala, K., Heinonen, H., and Schreck-Purla (1972). Uraemic waste recovery. Proc. Conf. EDTA, 9, 514-520.

Sparks, R. E., Saleman, R. M., Meier, P. M., Litt, M. H., and Lindon, O. (1972). Binders to remove metabolite from the GI tract. Trans. Am. Soc. Artif. Int. Org. 18: 458-464.

Sparks, R. E. (1979). Review of gastrointestinal perfusion in treatment of uraemia. Clin. Nephrol. 11:81.

Symmour, A. C., Philip, P. C. (1954). Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem. 209: 145.

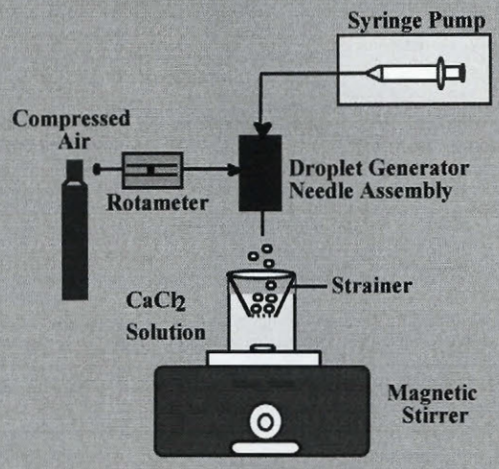
Walker, J. M., Jacobson, R. L., Stephen, W. J., and Rose, D. (1977). The role of adsorbents in the wearable artificial kidney. pp. 137-149. In: Gilchrist, T. (ed.), Artificial Org. Macmillan, London.

Wong, H. and Chang, T. M. S. (1991). The microencapsulation of cells within alginate polylysine microcapsules prepared with the standard single drop technique: Histologically identified membrane imperfections and associated graft rejection. Biomat. Artfi. Cells Immo. Biotechnol. 19: 687-697.

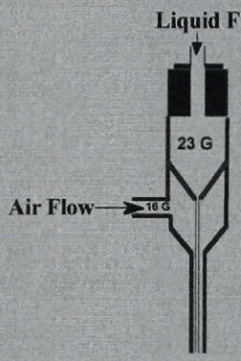
Wong, H. and Chang, T. M. S. (1991). A novel two step procedure for immobilization of living cells in microcapsules for improving xenograft survival. Biomat., Artifi. Cells Immo. Biotechnol. 19: 687-697.

Table 3.I. Effect of alginate concentration on microcapsule shape, diameter and cell viability. The initial number of the bacterial cell was 1.0×10^5/100 microcapsules.

Alginate concentration (% wt./vol.)	Microcapsule diameter (μm).	Microcapsule morphology (percent of spherical microcapsules.)	Viable cell in / 100 microcapsules after 24 hours of incubation.
1.00	455±55	10	3.62×10^6
1.25	395±68	50	3.43×10^6
1.50	380±73	75	3.32×10^6
1.75	430±80	98	3.35×10^6
2.00	500±45	98	3.52×10^6
2.25	490±80	98	3.12×10^6



A: Microencapsulation Apparatus



B: Droplet Needle Assembly

Figure 3.1: Microencapsulation Apparatus Assembly.

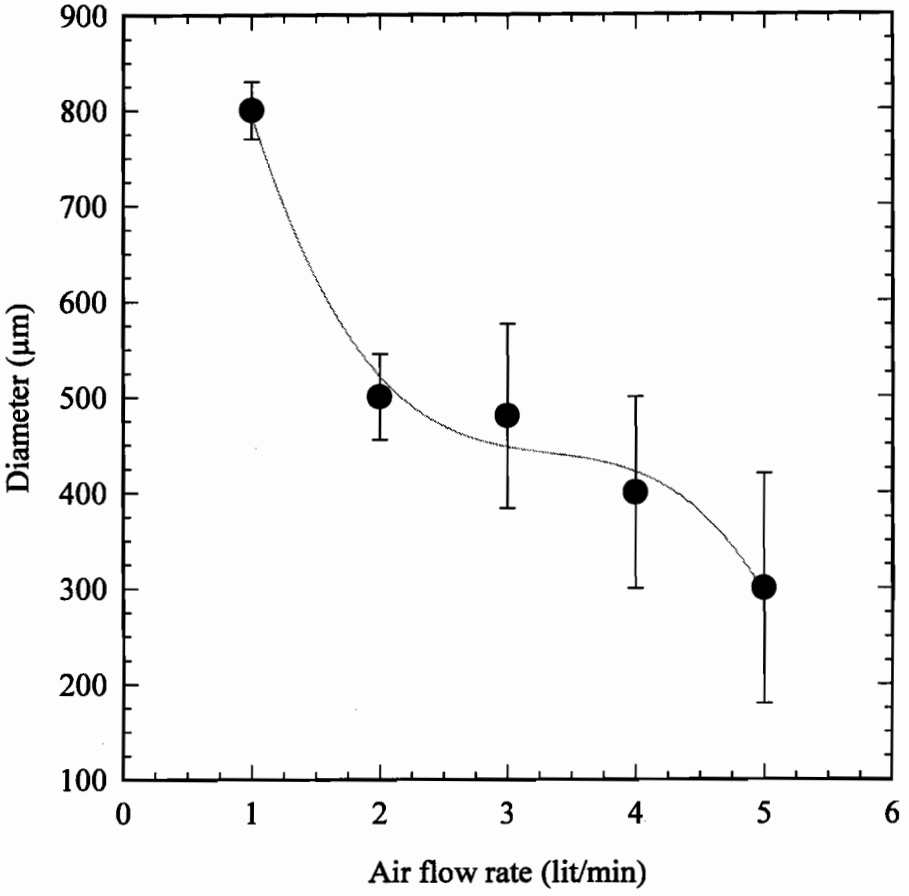


Figure 3.2: Effect of air flow rate on the microencapsulation process.

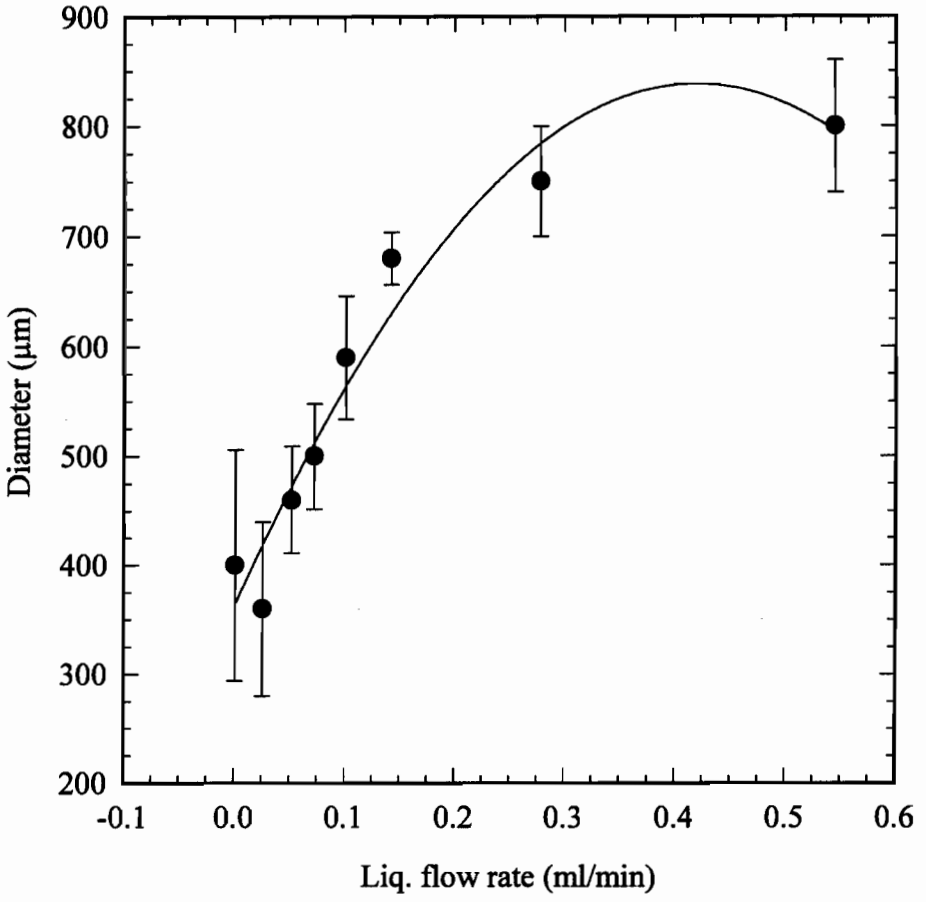


Figure 3.3: Effect of liquid flow rate on the microencapsulation process.

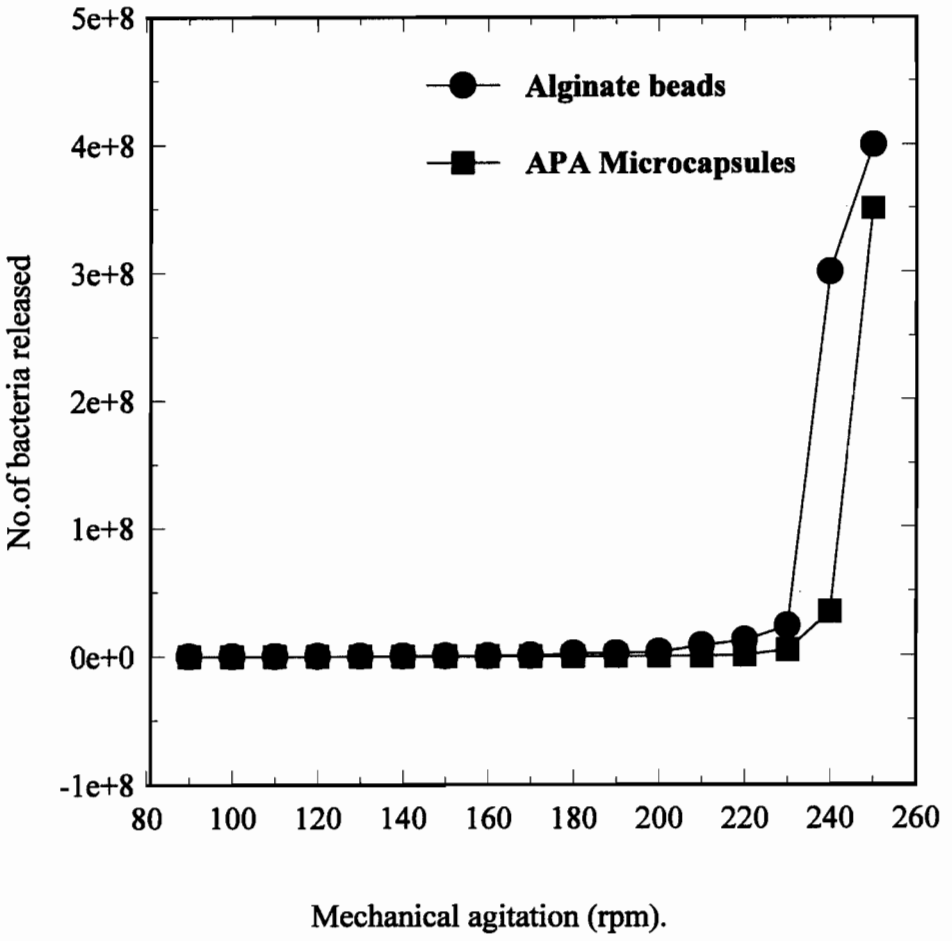


Figure 3. 4: Mechanical strength of alginate beads and APA-Microcapsules as a function of bacterial cells released.

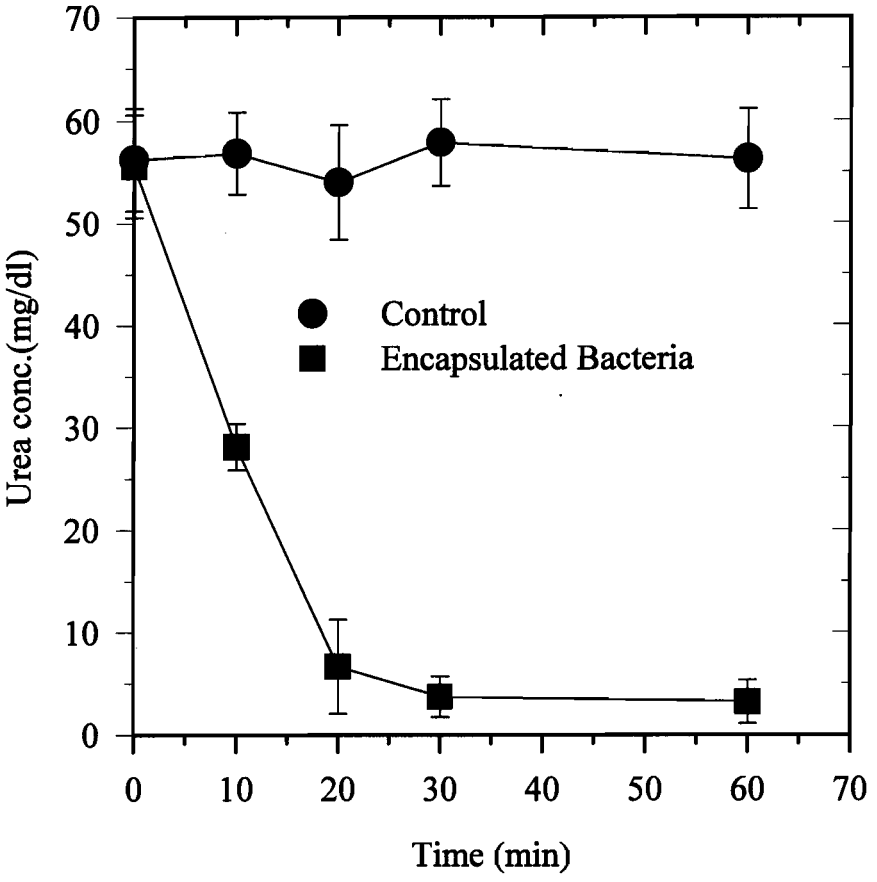


Figure 3.5: APA-Encapsulated bacteria on plasma urea removal.

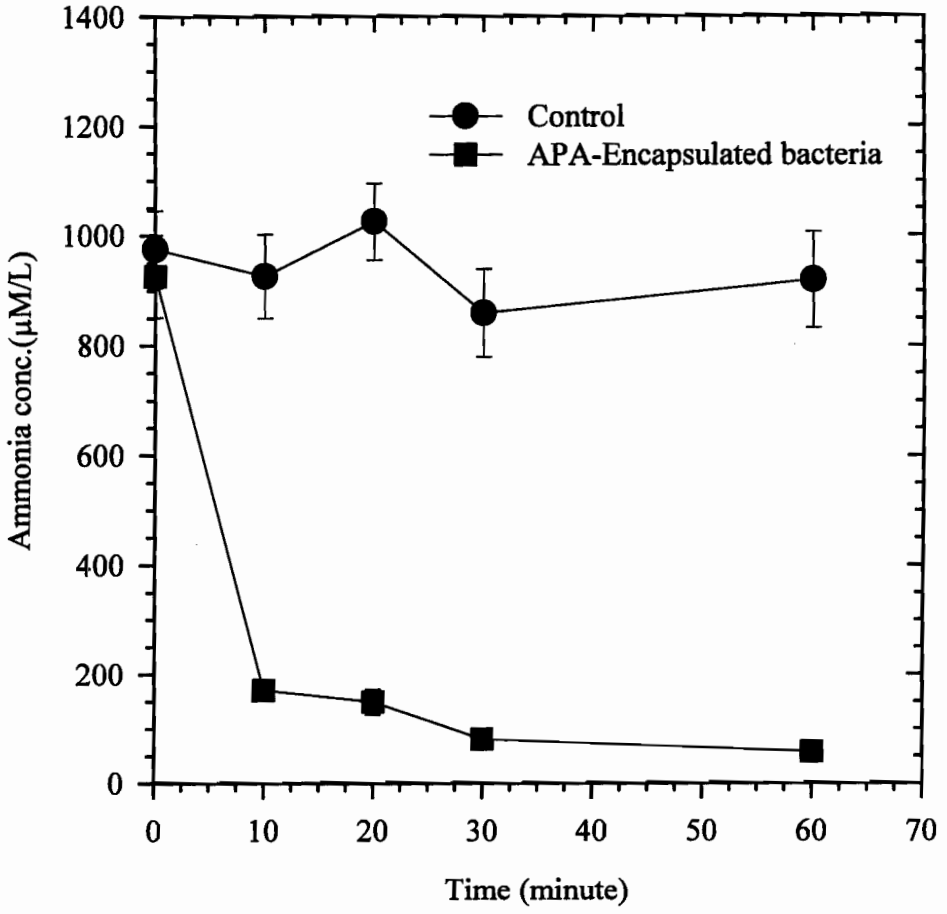


Figure 3.6: APA-Encapsulated bacteria on plasma ammonia removal.

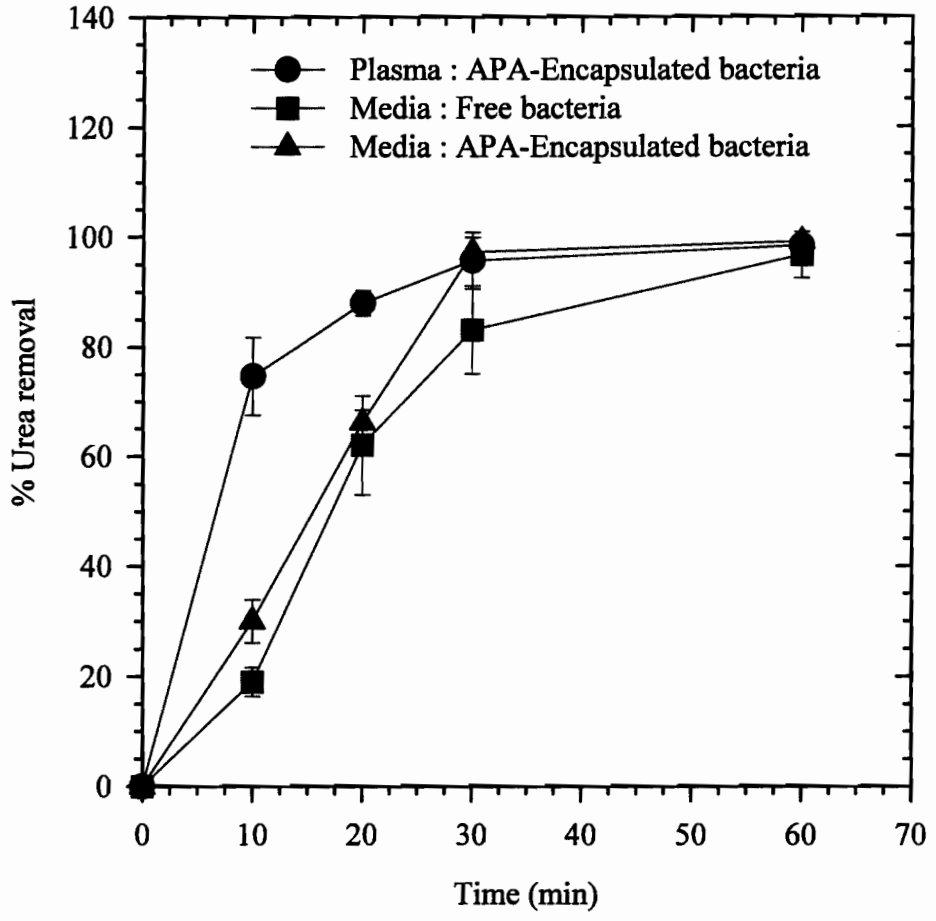


Figure 3.7: Comparative study of the urea depletion profile of free and APA-Enacpsulated bacteria in modified reaction media and APA-Encapsulated bacteria in plasma.

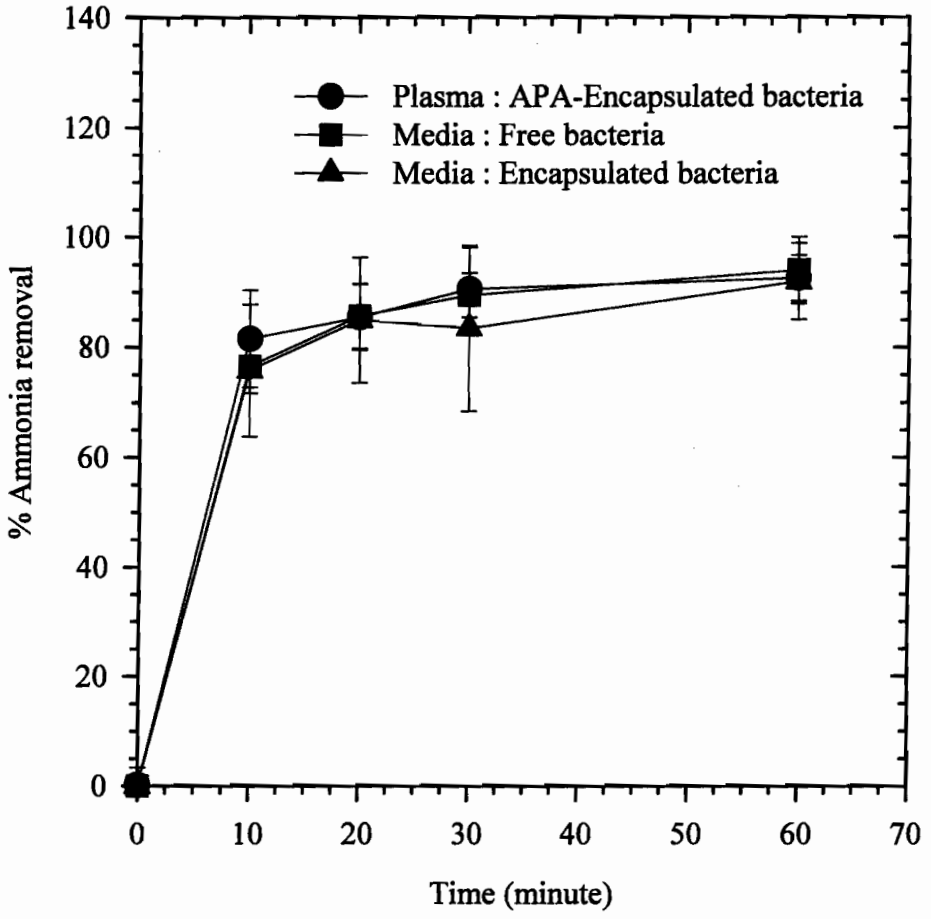


Figure 3.8: Comparative study of the ammonia depletion profile of free and APA-Enacpsulated bacteria in modified reaction media and APA-Encapsulated bacteria in plasma.

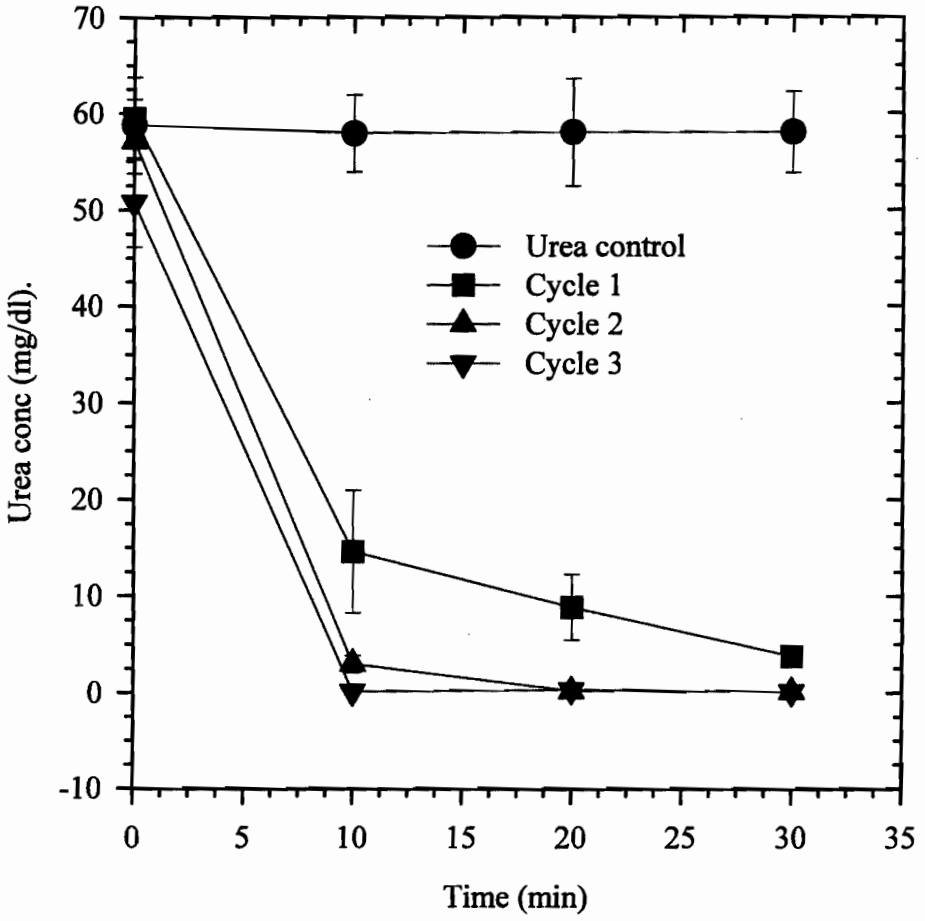


Figure 3.9: APA Microcapsules reused as a function of the plasma urea removal efficiency.

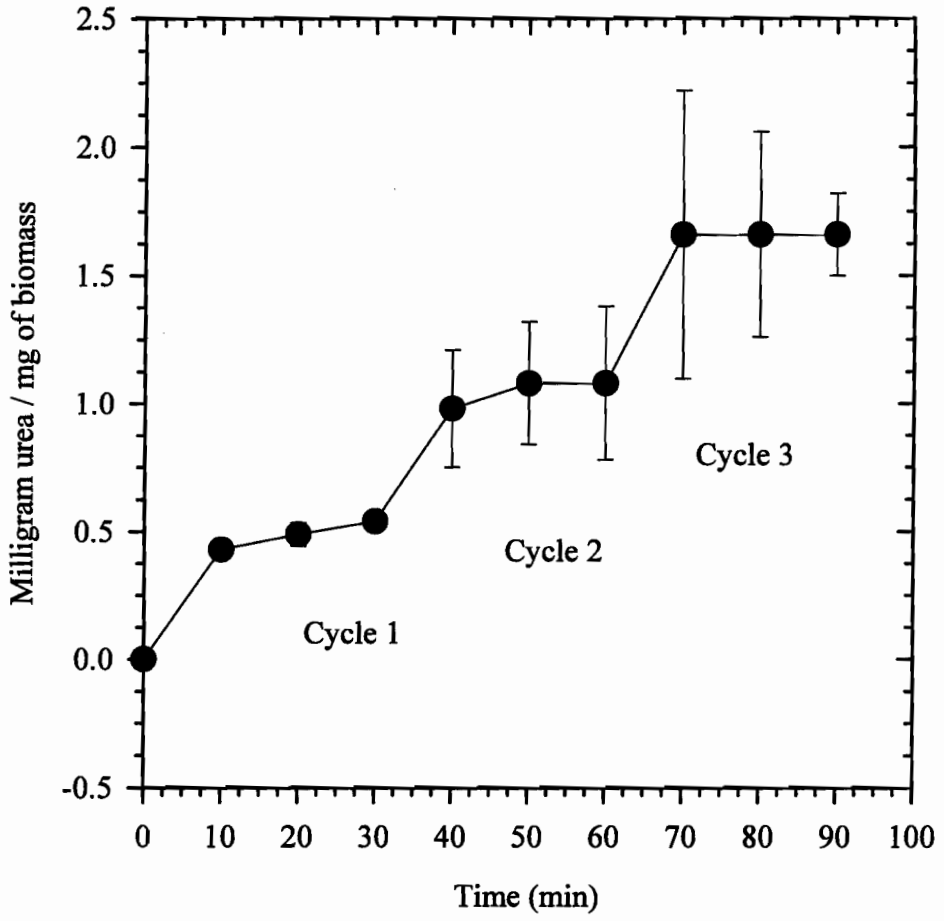


Figure 3.10: Cumulative removal of plasma urea by APA-Encapsulated bacteria.

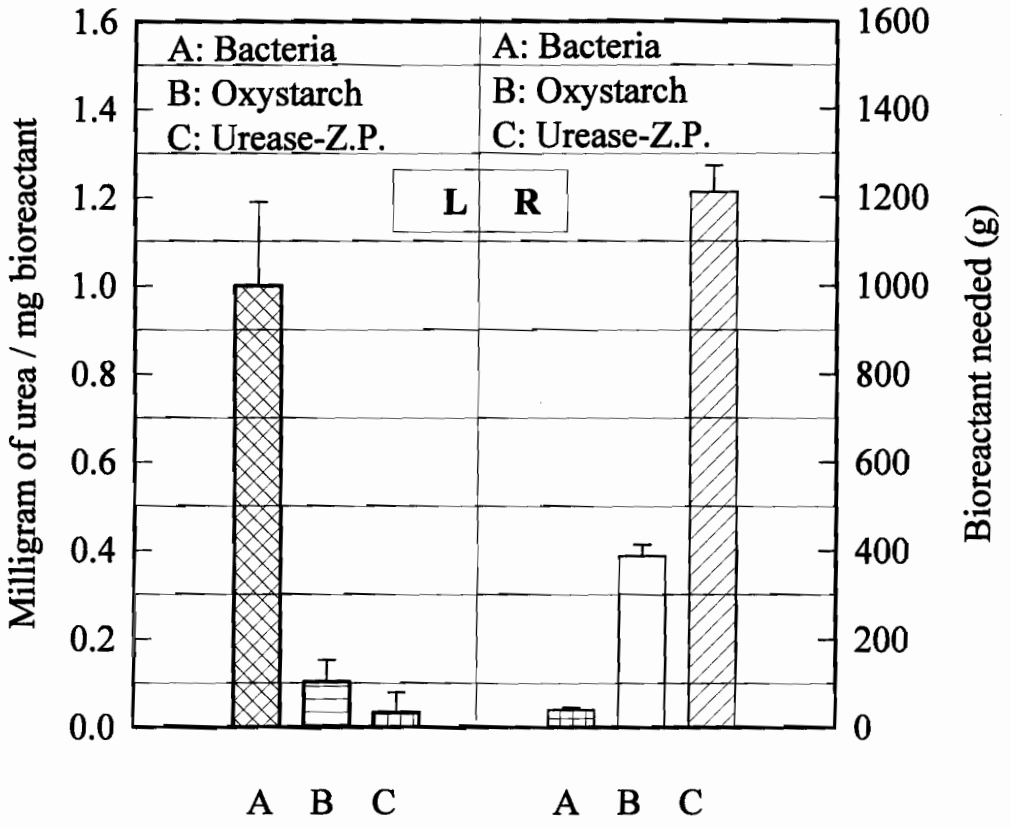


Figure 3.11: Comparative study.

L- Urea removal efficiency of APA-Encapsulated bacteria, oxystarch, and urease-Z.P.

R- Amount of bioreactant needed to lower urea concentration from 100 mg /dl to 6.86 mg/dl from the total 40 liter body fluid compartment in a 70-kg adult man.

APPENDIX 2

2.1 MICROCAPSULE MORPHOLOGY STUDY

2.1.1 OBJECTIVE

To evaluate the alginate-poly-L-lysine (APA) membrane condition and performance during in-vitro plasma urea and ammonia removal studies at different cycles of operations.

2.2 PROCEDURE

Used APA microcapsules were collected after each cycle of in-vitro plasma urea and ammonia removal studies. For this microcapsules were washed by autoclaved distilled water, placed in water and microscopy was performed. The size of the microcapsules were obtained by sizing with an eye peace micrometer attached to the microscope.

2.3 RESULTS AND DISCUSSIONS

Results (Figure 3.12) show a photomicrograph of freshly prepared APA microcapsules. A very firm APA membrane can be seen in the photomicrograph. Figure 3.13 shows the photomicrograph of microcapsules after cycle 1 of plasma

urea and ammonia removal studies. A slight growth of bacteria in side the membrane can easily be visualised. In cycle 2 (Figure 3.14), a slightly higher, compared to cycle 1, in bacterial cell number is observed. Figure 3.15 summarises the microscopic study of APA microcapsules after the cycle 3 reused and shows a considerable increase in bacterial cell number compared to cycle 1 and 2. When we proceed this study for cycle 4 (Figure 3.16), a large number of bacterial cells coming out of microcapsules were noticed. But even at this high growth of bacterial cells APA membrane were found intact (Figure 3.16). Based on this microscopic study and bacterial cell released studies described earlier (Figure 3.4), a recycle limit of three is recommended.

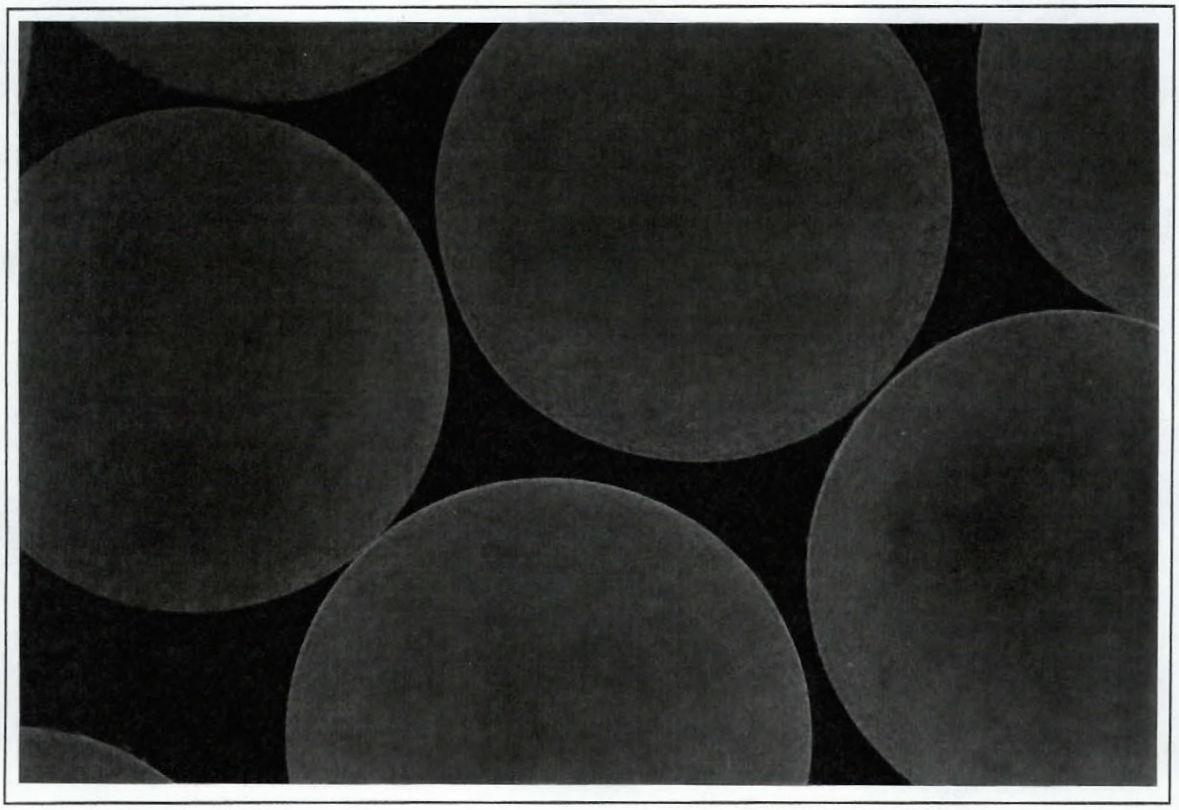


Figure 3.12: Photomicrograph of freshly prepared alginate-poly-L-lysine-alginate (APA) microcapsules (500 $\pm$ 45 μm) containing genetically engineered *E. coli* DH5 cells for urea and ammonia removal studies.

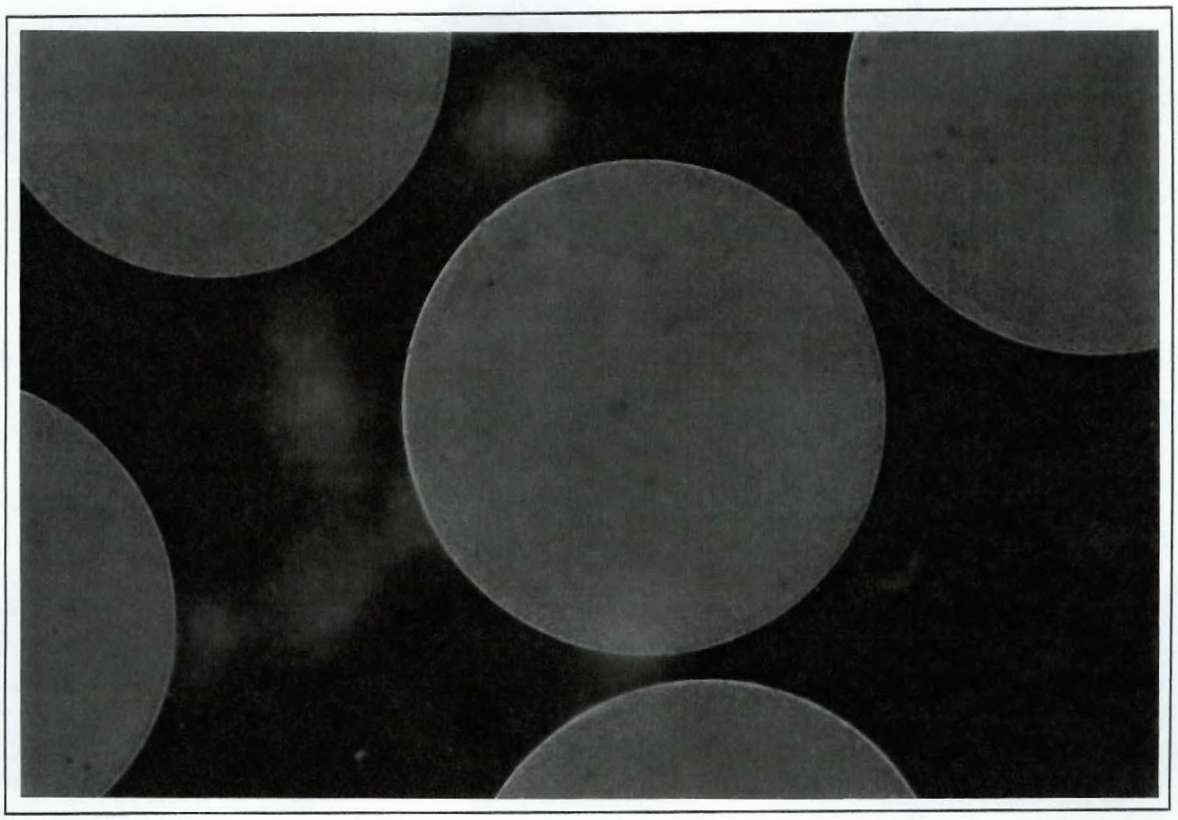


Figure 3.13: Photomicrograph of alginate-poly-L-lysine-alginate (APA) microcapsules containing genetically engineered bacteria *E. coli* DH5 cells after cycle 1 of in-vitro plasma urea ammonia removal studies.

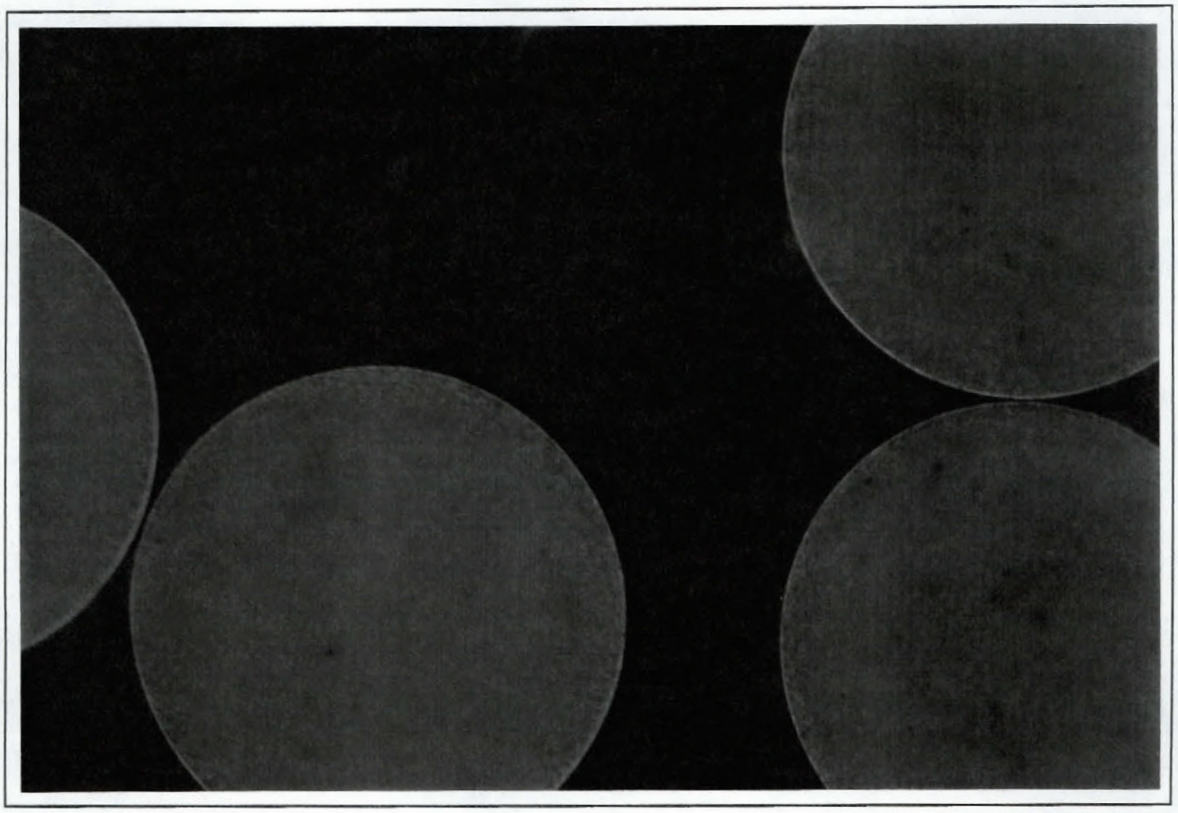


Figure 3.14: Photomicrograph of alginate-poly-L-lysine-alginate (APA) microcapsules containing genetically engineered bacteria *E. coli* DH5 cells after cycle 2 of in-vitro plasma urea and ammonia removal studies.

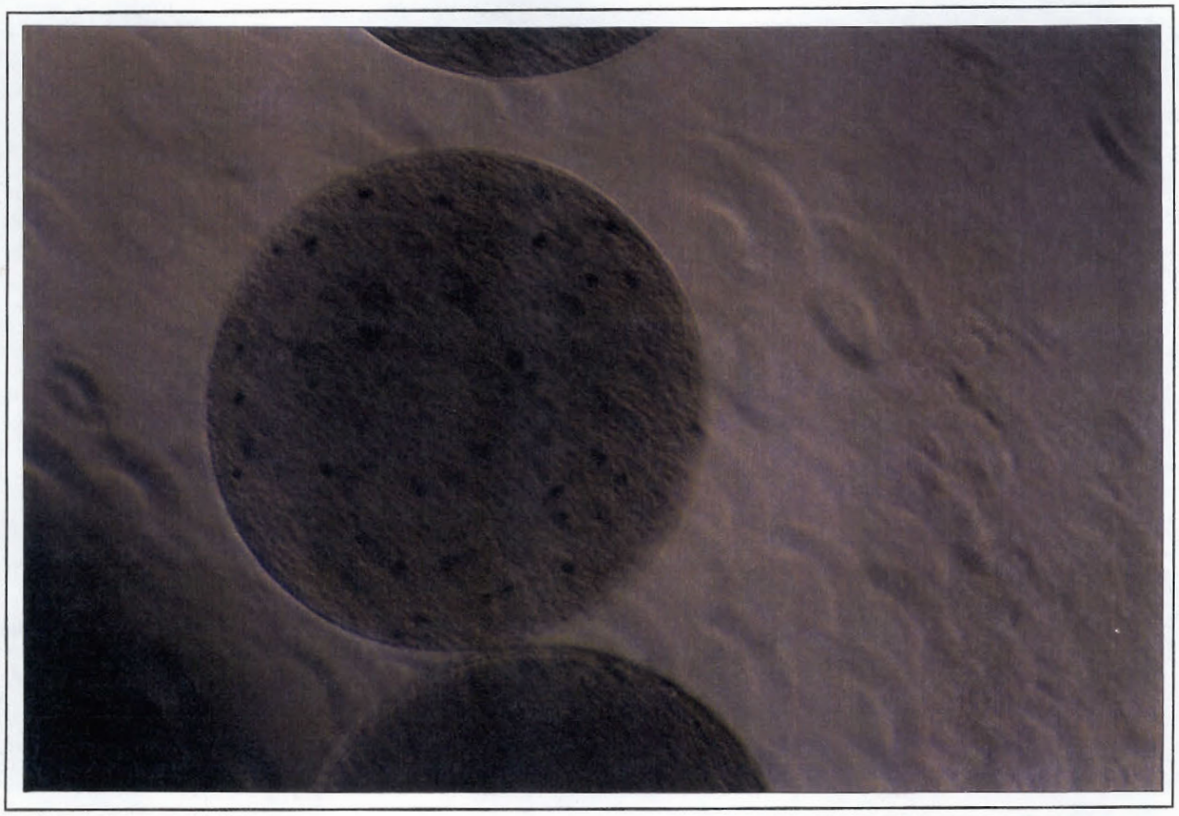


Figure 3.15: Photomicrograph of alginate-poly-L-lysine-alginate (APA) membrane microcapsules containing genetically engineered bacteria *E. coli* DH5 cells after cycle 3 of in-vitro plasma urea and ammonia removal studies.

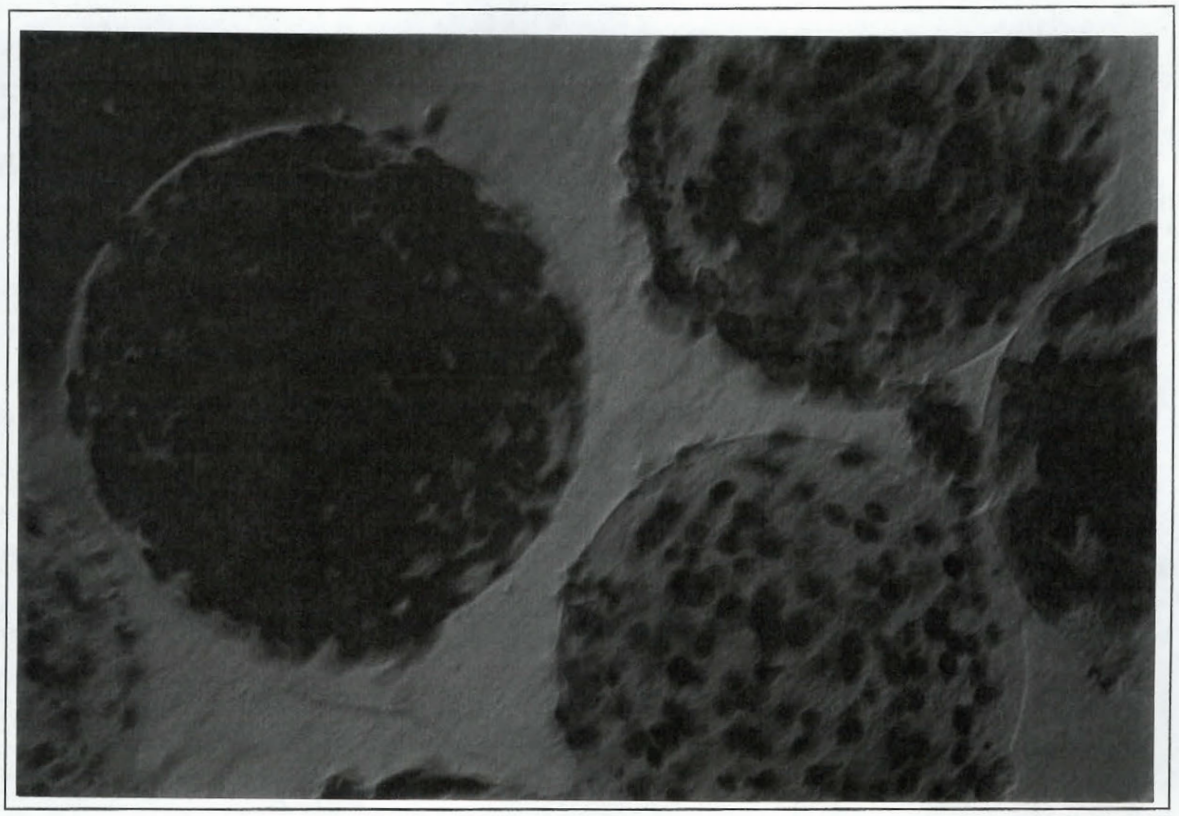


Figure 3.16: Photomicrograph of alginate-poly-L-lysine-alginate (APA) membrane microcapsules containing genetically engineered bacteria *E. coli* DH5 cells after cycle 4 of plasma urea and ammonia removal studies.

CHAPTER 4

Artificial Cells for Plasma Urea and Ammonia Removal using a Continuous Process Column Bioreactor containing Microencapsulated Genetically Engineered *E. coli* DH 5 Cells

S. Prakash and T.M.S. Chang

Accepted (along with other results) in Biomaterials Artificial Cells and Immobilization Biotechnology, an International Journal.

4.1 ABSTRACT

We report a novel approach for plasma urea and ammonia removal using artificial cells microencapsulating genetically engineered bacteria *E. coli.* DH5 cells in this article. This has been evaluated for use in a column bioreactor for removing plasma urea and ammonia. In 30 minutes, microencapsulated *E coli* DH 5 cells in a column bioreactor lowered the plasma urea concentration from 45.85 ± 2.98 mg.dl^{-1} to 10.47 ± 3.45 mg.dl^{-1}, and plasma ammonia concentration from 679 ± 32 μM.l^{-1} to 46.00 ± 4.00 μM.l^{-1}. The efficiency of this bioreactor for plasma urea and ammonia removal is comparatively very high than the other available methods. Initial plasma urea and ammonia concentration do not effect the plasma urea and ammonia removal efficiency of the column bioreactor.

Key Words: artificial cells, column bioreactor, microencapsulation, urea, ammonia, alginate-poly-l-lysine-alginate (APA), genetically engineered bacteria.

4.2 INTRODUCTION

In the past two decades, molecular technology has greatly influenced the direction of biomedical research (Griffin and Griffin,1995; Mange,1984; Carmen, 1992). Potential medical applications include discovering the genetic basis for diseases, gene therapy, production of therapeutic agents and many others (Griffin and Griffin,1995; Mange,1984; Carmen, 1992). There are numerous genetically engineered microorganisms with many unique properties (Griffin and Griffin,1995; Mange,1984; Carmen, 1992). However, the use of genetically engineered microorganisms in medicine is mainly for the production of important therapeutic agents and proteins. This is because they cannot be safely

introduced into the body for potential applications. In this article we report our assessment to combine the use of artificial cells (Chang, 1964) with genetically engineered microorganisms. The example of *E. coli* DH5 cells for the removal of plasma urea and ammonia is used as an example for this feasibility study.

Removing urea and ammonia from the plasma is often necessary in kidney failure and liver failure (Chang, 1967,1978; Giordino et al., 1975,1976; Guyton, 1995). Presently, we do not have a good system for the removal of urea and ammonia (Chang, 1994; Friedman, 1995; Prakash and Chang, 1993, 1995). Currently used methods, for example dialysis, hemofiltration and use of physio-chemical adsorbents, are not effective for the removal of urea (Chang, 1972; Ilan and Chang, 1972; Giordino et al., 1975,1976; Friedman, 1995). Further, dialysis and hemofiltration techniques are complex and expensive (Chang, 1994; Giordino et al., 1975,1976). Similarly, the use of adsorbents is associated with many problems (Giordino et al., 1975,1976). For example, oxystarch is associated with a high dose requirement and pH selective urea removal capacity (Chang, 1981, 1992, 1994; Friedman, 1978,1995; Giordino et al., 1975,1976). Several improvements, therefore, have been examined (Chang, 1976, 1978; Friedman, 1978, 1995; Giordino et al., 1975,1976). This includes the use of microencapsulated reactants (Chang, 1967,1971,1972,1978; Esposito, 1980; Friedman, 1978, 1995; Hughes et al., 1981; Juggi, 1973; Kjellstrand et al., 1981; Kolf, 1990; Nelson, 1962; Man, 1976; Sparks, 1972,1979), encapsulated enzyme urease to convert urea into ammonia that is subsequently removed by the coencapsulated ammonia adsorbent (Chang, 1967,1971, 1972, 1978; Cataneo and Chang, 1991), microencapsulated multi-enzyme complex to convert urea and ammonia into essential amino acids (Gu and Chang, 1991,1992), and the use of lyophilized urea eating soil bacteria (Setala, 1972; Setala et a., 1973) and several others. Despite all research we still do not have a suitable urea and ammonia removal system (Chang, 1993; Friedman, 1995; Prakash and Chang, 1995).

In search to find a suitable alternative system for urea and ammonia removal we were the first to suggest the possibility of using a biotechnological approach using the

combination of the concept of artificial cells and genetic engineering (Chang, 1995; Chang and Prakash, 1995; Prakash and Chang 1993,1995). In earlier in-vitro batch bioreactor studies, we have shown that the microencapsulated genetically engineered bacteria *E. coli* DH 5 cells have a 20-30

fold greater efficiency for plasma urea and ammonia removal when compared to the presently used methods (Chang and Prakash, 1995; Prakash and Chang, 1993, 1995). In this article we report our evaluation of using a column bioreactor for the plasma urea and ammonia removal.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

Alginic acid (low viscosity, lot 61194) from Kelco and poly-L-lysine (MW 16100, lot 11H5516) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were of analytical grade.

4.3.2 Microorganism

The bacterial strain used was *Escherichia coli* DH5, containing the *K. aerogen* urease gene, was provided by Prof. R.P. Haussinger (Mobley et al., 1989; Scoot et al., 1989).

4.3.3 Culture Media

4.3.3.1 Synthetic Media I

This medium, commercially known as Lauria-Bertani (LB), was used for biomass

growth before microencapsulation in alginate beads and for the experiment with free cells. Its composition was as follows in doubled distilled water: bactotryptone (Difco), 10.00 ($g.l^{-1}$); bactoyeast extract (Difco), 5.00 ($g.l^{-1}$); sodium chloride (BDH), 10.00 ($g.l^{-1}$). Its pH was adjusted to 7.5 using about 1.00 ml of 1.0N NaOH.

4.3.3.2 Synthetic Media II

This medium was used for in-vitro media urea and ammonia removal experiment, mimicking the uremia patients plasma compositions (Castelman et al., 1974). Its composition was as follows in sterile double distilled water: glucose (BDH), 1.0 ($g.l^{-1}$); magnesium sulphate, 0.002 ($g.l^{-1}$); dipotassium monohydrogen phosphate, 0.03 ($g.l^{-1}$); vitamin B_{12}, 0.007. Each component of this media, except vitamin B_{12} which was filtered sterilised, was sterilised separately using Castel Labclave for 15 min at 250 ^{0}F. Filter sterilised urea was added to this media to make the urea concentration 100 mg. l^{-1}.

4.3.3.3 Natural Media

This media was bovine plasma. This media was used for the plasma urea and ammonia removal studies and filter sterilised urea was added to it. This was done to mimic the uremic patient's plasma characteristics in-vitro (Castelman et al., 1974).

4.3.4 Microencapsulation Technique

Bacterial cells were grown in the synthetic media II in 250 ml Erlenmeyer flasks at 37 ± 1.0 ^{O}C, and 100 rpm, using the standard primary shake flask cultures grown to stationary phase (overnight) inoculate, in a Labline orbital environ-shaker. Following centrifugation in a Sorval at 10,000 g for 20 min at 4 ^{0}C the bacterial cells were harvested. The obtained cell mass was then washed for five times to remove media components and used for microencapsulation. The details of the microencapsulation procedure and the process variables were described elsewhere (Prakash and Chang, 1995). Very briefly,

bacterial cells were suspended in an autoclaved 2 % sodium alginate in 0.9 % sodium chloride solution. The viscous alginate bacterial suspension was pressed through a 23-gauge needle using syringe pump (Compact Infusion Pump Model 975, Harvard App. Co., MA). Compressed air through a 16-gauge needle was used to shear the droplet coming out of the tip of the 23-gauge needle. The droplet was allowed to gel for 15 min in a gentle stirred ice-cold solution of 1.4 % calcium chloride. After gelation the bacterial alginate gel beads were coated with poly-L-lysine (0.05 % in HEPES, pH 7.20) for 10 minutes. The beads were then washed with HEPES and coated with 0.01 % alginate solution for 4 minutes. The alginate-poly-L-lysine alginate microcapsules were washed in a 3 % citrate bath (3.0 % in 1:1 HEPES buffer saline, pH 7.2) to liquefy the gel in the microcapsules. The microcapsules formed were stored at 4 ^{0}C and used for the experiments. The diameter of obtained APA microcapsules were 500 $\pm$ 45 μm.

4.3.5 Continuous Urea and Ammonia Removal Process

4.3.5.1 Bioreactor Design and Apparatus Set-up

The device (Figure 4.1) consisted of a 2 ml glass syringe, called column bioreactor (Jhons Scientific Inc. Montreal, Canada). The column is connected to a 100 ml conical flask containing either synthetic media or plasma as shown in figure 4.1. Microcapsules were retained into the column between two perforated 192.4 μ pore size membrane. A peristaltic pump (Minpuls 2; Gilson, Mandel Scientific Ltd., Canada) was used to circulate either the synthetic media or plasma through the column from the bottom to top fluidizing the bed of the microcapules. The flow rate of this pump was designated as the fluridization rate F (l h^{-1}). An autoclaved Teflon membrane was used to make the current from the reservoir media or plasma to the column bioreactor through the peristaltic pump. The dilution rate (D) is defined as the ratio of the feeding rate (Q) to the total liquid volume V_L (Taillandier et al., 1994). The residence time (t) is the inverse of the dilution rate.

4.3.6.2 Operational Conditions

The column and the Teflon tubing were sterilised using Castel Labclave at 250 ^{0}C for 15 min. Microcapsules containing 5 mg of genetically engineered bacteria were retained in the column bioreactor with either suitable media or plasma. The complete apparatus set-up and experimental operations were performed at room temperature and inside a U.V. sterilization chamber (Canadian Cabinet Ltd.) to maintain the sterility of the complete process.

4.3.6 Analytical Methods

4.3.6.1 Urea Determination

Urea concentrations were determined using a BUN kit (Sigma Chemicals Co., St. Louis, MO); which is based on a quantitative colorimetric method and widely used for the determination of blood urea nitrogen (BUN) in plasma or serum (Sigma, manual BUN KIT).

4.3.6.2 Ammonia Determination

Ammonia was analysed using a fluorescent light scattering Multistat III microcentrifuge analyser. This was based on the reductive amination of 2-oxoglutarate, using glutarate dehydrogenase (GLDH), and reduced nicotinamide adenine dinucleotide (NADH). The decreased in absorbance at 340 nm due to oxidation of NADH is proportional to the ammonia concentration. A separate standard curve, for each set of ammonia measurement, was prepared using ammonium sulphate as ammonia source and a counter control of Sigma supplied standard ammonia solution.

4.4 RESULTS AND DISCUSSION

4.4.1 Bioreactor Design and Operation

We first assayed the fermentation by microencapsulated genetically engineered *E. coli* DH5 cells for the urea and ammonia removal in a batch reactor (Chang and Prakash, 1995; Prakash and Chang, 1993,1995). This gave us basic parameters for urea and ammonia removal efficiency, microcapsule stability, and its recycle feasibility (Chang and Prakash, 1995; Prakash and Chang, 1993,1995). However, batch reactors can not be conveniently used for therapeutical applications. We therefore, selected a column bioreactor simulating the fluidized bed bioreactor (Figure 4.1). The objective of these experiments was to evaluate the performance of the bioreactor for urea and ammonia removal from the plasma.

4.4.2 Performance of Continuous Process Column Bioreactor for Removal of Plasma Urea

Experiments were designed to explore the possibility of using microencapsulated genetically engineered bacteria in a column bioreactor to lower the plasma urea concentration. The plasma used for this study had a urea concentration of 45.843 ± 2.91 $mg.dl^{-1}$. In the experiment, the plasma was passed through the bottom of the column containing 5 mg of encapsulated bacteria with a steady flow rate of 35.40 $ml.h^{-1}$. Figure 4.2 summarises the results of the experiment. It is found that using this column bioreactor one can lower the urea concentration about 83.57 % in 20 min, 92.12 % in 30 min and 98.02 % in 60 min. When we compare this efficiency with the currently used methods for urea removal, we found this biotechnological approach has several fold greater urea removal efficiency (Giordano et al., 1975, 1976; Friedman, 1978; Sparks et al.,1972,1979).

4.4.3 Effect of initial Plasma Urea Concentration on Urea Removal Performance of the Continuous Column Bioreactor

We also evaluated the column bioreactor performance for plasma urea removal using different initial plasma urea concentrations. This was done by challenging the same bioreactor to the randomly selected plasma containing urea concentration of 45.84, 100.14, 115.26 and 186. 34 $mg.dl^{-1}$. This was done to evaluate if one can use this column bioreactor for different degree of uremic patients. Results of these experiments were illustrated in figure 4.3. A slower rate at higher plasma urea concentration was observed. However, a detailed study to prepare a generalised standard measurement to evaluate the correlation between bacterial cell loading in the column and the multiple plasma urea concentration is required.

4.4.4 Performance of Continuous Process column Bioreactor for Removal of Plasma Ammonia

The following study was aimed to evaluate the potential of this column bioreactor for the removal of plasma ammonia. Figure 4.4 shows the experimental results of plasma ammonia removal. When challenged the column bioreactor containing APA membrane microencapsulated genetically engineered bacteria plasma ammonia concentration fell down from 741 $\pm$ 25.00 to 26.96 $\pm$ 5.78 μM in 30 minutes. This is a 96.36 % reduction in plasma ammonia level in only in 30 minutes. This efficiency of lowering plasma ammonia is very fast compared to other approaches.

4.4.5 Effect of initial Plasma Ammonia Concentration on Ammonia Removal Performance of the Column Bioreactor

We also studied the influence of plasma ammonia concentration on column bioreactor performance (Figure 4.4). For this experiment, an initial ammonia concentration of 462 $\pm$ 8.26 μM, 741 $\pm$ 26.12 μM, and 985 $\pm$ 34.16 μM was selected. And to prepare

these ammonia concentrations, the desired amounts of ammonium sulphate was added to plasma. Results (Figure 4.5) show that rate of ammonia removal is greater when the ammonia concentration is lower.

4.4.6 Operational stability of Microencapsulated Bacteria in Column -cycle Study

Bioreactor containing microencapsulated genetically engineered *E. coli* DH5 cells were tested for their operational stability for urea removal. The microcapsules were packed into a column and challenged to experimental uremic plasma several times. The performance of the column bioreactor, in terms of urea removal efficiency, is shown in figure 4.6. Results show that the column is increasingly effective. This is because of the dynamic nature of the bioreactor as bacterial cells are continuously growing. When we compare this with our in-vitro results (Chang and Prakash, 1995; Prakash and Chang, 1993, 1995) and did morphological study we find it is safe to use this bioreactor for three cycles only.

4.5 CONCLUSIONS

We show the feasibility of using a column bioreactor containing genetically engineered bacteria *E. coli* DH5 for removing urea and ammonia from plasma in-vitro. Using microencapsulated genetically engineered bacteria it is feasible to reduce plasma urea and ammonia levels from 45.84 ± 2.91 mg.dl^{-1} to 10.24 ± 2.60 mg.dl^{-1} and 741 ± 25.00 to 26.96 ± 5.78 μM in 30 minutes. Very high plasma ammonia removal using this column bioreactor is achieved. However, at a very high concentration of plasma urea and ammonia the removal efficiency of the column was found to be slower. This has implications on the removal of urea or ammonia from plasma in plasma separation procedures like plasmapheresis or from the dialysate. However, our experiments only show the feasibility of this column bioreactor, because much further detailed study, especially

safety study, is required. And, if proven safe, this approach might be extended to other types of genetically engineered cells for the removal of other unwanted metabolites.

4.6. ACKNOWLEDGEMENTS

TMSC gratefully acknowledges the research support of the Medical Research Council of Canada and the Virage "Centre of Excellence in Biotechnology" support of the Quebec Ministry of Higher Education Science and Technology. SP acknowledges the support of a National Overseas Scholarship from the Government of India. The generous gift of the *E. coli* DH5 by Prof. R.P. Haussinger is gratefully acknowledged. Mr. Colin Lister's technical assistance is also acknowledged.

4.7 REFERENCES

Carmen, A.M. (1992). Am. J. of Human Genetics. 50, 245-260.

Castelman, B. and Betty, U. McNeely. (1974). Case records of the Massachusetts general hospital. New Engl. J. of Med., Vol. 290 No.1, 39-49.

Cattaneo, M. and Chang. T.M.S. (1991). The potential of microencapsulated urease-zeolite oral sorbent for the removal of urea in uraemia. TASAIO-official J. of American Society of Artificial Internal Organs. 37, 80-87.

Chang, T.M.S., and Poznansky M.J. (1968). Semipermeable microcapsules containing catalase for enzyme replacement in acatalasaemic mice. Nature (London), 218, 243-245.

Charls, S., David, M., and Stacia, M. (1990). pp. 526-527. In : Deborah, A. (ed.), Human physiology : foundations and frontiers.

Chang, T.M.S. (1964). Semipermeable Microcapsules. Science. 146 (3643), 524-525.

Chang, T.M.S. (1969a). Removal of endogenous and exogenous toxins by a microencapsulated adsorbent. Can. J. Physiol. Pharmacol., 47(12), 1043-1045.

Chang, T.M.S., and Loa, S.K. (1970). Urea removal by urease and ammonia absorbents in the intestine. The Physiologist, 13(3), 70.

Chang, T.M.S. (1971). The in-vivo effects of semipermeable microcapsules containing L-asparaginase on 6C3HED lymphosarcoma. Nature, 229, 117-118.

Chang, T.M.S. (1972a). Artificial Cells. Thomas, Springfield, Illinois.

Chang, T.M.S. (1972 b). Hemoperfusion over microencapsulated adsorbent in a patient with hepatic coma. Lancet II, 1371-1372.

Chang, T.M.S., and Migchelsen, N. (1973). Characterisation of possible toxic metabolites in uremia and hepatic coma based on the clearance spectrum for larger molecules by the ACAC microcapsules artificial kidney. Trans. Am. Soc. Artif. Intrn. Organs. 19, 314-318.

Chang, T.M.S. (1976). Hemoperfusion alone and in series with ultrafiltration or dialysis for uremia, poisoning and liver failure. Kid. Int., 10, S305-S311.

Chang, T.M.S. (1978). Artificial Kidney, Artificial Liver, and Artificial Cells. Plenum Press, New York.

Chang, T.M.S., Malouf, C., and Resurreccion, E. (1979). Artificial cells containing multienzyme systems for the sequential conversion of urea into ammonia, glutamate, then alanine. Artif. Org., 3, S284-S287.

Chang, T.M.S. (1983). Artificial liver support : Requirement and approaches. Int. J. of Artif. `Organs. 6(4), 178-182.

Chang, T.M.S. (1984). Liver support systems. In : "Life support system in intensive care", Bartelett et al. (ed.), Year Book Med. Publ., Chicago. 461-485.

Chang, T.M.S. (1985a). Biotechnology of artificial cells including application to artificial organs. In comprehensive biotechnology : the principle applications & regulation of biotechnology in industry agriculture and medicine. pp. 53-73. In : Murray Moo Young (ed.), Pergamon press, N.Y.

Chang, T.M.S. (1985b). Biotechnology of artificial cells including application to artificial organs. In Comprehensive Biotechnology (Moo-Young, M., ed.), Vol.4, pp.53-72,

Pergamon Press, New York.

Chang, T.M.S. (1987). Recycling of NAD(P) by multienzyme systems immobilized by microencapsulation in artificial cells. In Methods in Enzymology (Mosbach, K., Ed.), Vol.136, pp.67-82, Academic Press, New York.

Chang, T.M.S. (1988). Medical applications of immobilized proteins, enzymes, and cells. In Methods in Enzymology (Mosbach, K., ed.), Vol.137, pp.444-457, Academic Press, New York.

Chang, T.M.S. (1988). Methods in the medical application of immobilized proteins enzymes and cells. Methods in Enzymology. 137, 444-457.

Chang, T.M.S. (1990). Biotechnological and medical application of immobilization of hepatocytes, microorganisms and enzyme systems by microencapsulation in artificial cells. Enzyme Engineering. 10(613), 331-339.

Chang, T.M.S. (1993). Living cells and microorganisms immobilized by microencapsulation inside artificial cells. pp. 183-194. In: Goosen, M.F.(ed.), Fundamentals of animal cell encapsulation and immobilization. CRC press, N.Y.

Chang, T.M.S. (1994). Artificial cell including blood substitute and biomicroencapsulation. Artificial Cells, Blood Substitute, and Immobilization Biotechnology, 22(5), vii-xiv.

Chang, T.M.S. (1995). Artificial cells with emphasis on bioencapsulation in biotechnology in Annual Review of Biotechnology (M.R. El-Gewely (ed.), Elsevier Science B.V.

Chang T.M.S. and Prakash, S. (1995). Artificial cells and genetically engineered microencapsulated for *E. coli* cells for urea and ammonia removal : Methods in Molecular Biology, Volume on "Expression and Detection of Recombinant Gene". Humana Press, USA Chapter 75 (in press).

Dayton, Leigh (1989). Cells by design, New Scientist, 3 June, 42-43.

Drukker, W., Parsons, F.M., and Mahar, J.F. (1983). Replacement of renal functions by dialysis. Martin, Nijhoff Publ. (Boston).

Esposito, R. Carmelo, G. (1980). Polyhalides. pp.133-153. In : Giordano Caramelo (ed.), Sorbents and their clinical applications. Academic press, London.

Friedman ,E. A. (1978). pp. 521-528. In : Eli, A. Friedman (ed.), Strategy in renal failure. Wily & Sons, N.Y.

Gardner, D. L., Kjellstrand, C. M., Fink, D.J., and Emmerling, D.C. (1984). Oral administration of microencapsulated urease and zirconium phosphate. Artif. Org., 8, 110.

Giordano, C., Esposito, R., and Pluvio, M. (1975). Oxycellulose and ammonia-treated oxystarch as insoluble polyaldehyde in uremia. Kid. Int., 7, S380-S382.

Giordano, C., Esposito, R., and Pluvio, M. (1976). Further studies with oxystarch. Kid. Int., 10, S266-S268.

Griffin , A.M. and Griffin, H.G. (1995). In: Molecular Biology : current innovations and future trends part 2, Oct., Horizon Scientific Press, England.

Gu, K.F. and Chang, T.M.S. (1988). Conversion of alpha-ketogluterate into L-glutamic acid with urea as ammonia source using multienzyme system and NAD immobilized by microencapsulation with artificial cell in bioreactor. Biotechnol. Bioengg. 32: 363-368.

Gu, K.F. and Chang, T.M.S. (1991). Conversion of urea or ammonia in to essential amino acids, L-leucine, L-valine and L-isoleucine using artificial cells containing an immobilized multienzyme system and dextran-NAD. Yeast alcohol dehydrogenase for enzyme recycling. J. Biotech. Applied Biochem. 12, 227-236.

Hughes, R., and Williams, R. (1981). Clinical experience with charcoal and resin hemoperfusion. Seminars in Liver Disease. 6(2),169-172.

Juggi, J.S. (1973). Extracorporeal cation-exchange circuits in the treatment of hyperammonemia of liver failure. Med. J. Aust., 1, 926-930.

Kjellstrand, G., Borger, H. Pru, C.G. and Fink, D. (1981). Trans. Am. Soc. of Artificial Internal Organs. 27, 24-30.

Kolff, W.J. (1990). Artificial kidney and artificial heart : future considerations. Int. J. Artificial Organs. 13(7), 404-406.

Kwok, W.Y., Kiparissides, C., Yuet, P., Harris, T.J. and Goosen, M.F. (1991). Mathematical modelling of protein diffusion in microcapsules : a comparison with experimental results. The Canadian Journal of Chemical Engineering, 69, 360-370.

Man, N.K., Drueke,T., Becker,A., Zingraff,J., Jungers,P., and Brentano, J-L.F. (1976).

Clinical use of oxystarch. Kid. Int., 10, S269-S272.

Mange, Arthur P. (Nov. 1984). In: Applied Genetic Engineering; future trends and problems, Noyes Publs., Bioscience, 34, 642.

Mobley, H.L. and Haussinger, R.P. (1989). Microbial ureases : significance, regulation, and molecular characterisation. Microbiolo. Rev., 53(1), 85-108.

Mosbach, K., and Mosbach, R. (1966). Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their applications in column techniques. Acta Chem. Scand., 20, 2807-2810.

Mueller,P., and Rudin, D.O. (1968). Resting and action potentials in experimental bimolecular lipid membranes. J. Theor. Biol., 18, 222-258.

Nealon, T.F., Jr., Ching, N. (1962). An extracorporeal device to lower blood ammonia levels in hepatic coma. Trans. Am. Soc. Artif. Intern. Org., 8, 226-228.

Pat, M.L., Kong, H.L., and Yew, S.S. (1993). Covalent Immobilization of aminoacylase to alginate for L-phenylalanine production. Chem. Tech. Biotech. 58, 65-70.

Prakash, S. and Chang, T.M.S. (1993). Microencapsulated genetically engineered *E. coli* cells containing genes form *K. aerogens* for urea and ammonia removal. Biomat. Artifi. Cells & Immob. Biotechnol., 19(4), 687-697.

Prakash, S. and T.M.S. Chang (1995). Preparation and in-vitro analysis of genetically engineered *E. coli* cells, microencapsulated in artificial cells for urea and ammonia removal : Biotechnology and Bioengineering, Vol. 47, 629-626.
John Willy and Sons USA.

Prakash, S. and T.M.S. Chang (1996). A new approach of using artificial cells containing genetically engineered *E. coli* DH5 cells for urea and ammonia removals. Submitted ASAIO journal.

Scoot, B., Mulrooney, H. Stauart, P. and Haussinger, R.P. (1989). Regulation of gene expression and cellular localization of cloned *K. aerogens* urease. J. of Gen. Microbiol., 135, 1769-1776.

Setala, K., Heinonen, H. and Schreck-Purla (1972). Uremic waste recovery. Proceeding Conf. E.D.T.A., 9, 514-520.

Setala, K., Heinonen, H. and Schreck-Purla (1973). Ingestion of lyophilized soil bacteria for

alleviation of uremic symptoms. J. Int. Res. Comm. System. 73.

Sparks, R.E. (1979). Review of gastrointestinal perfusion in treatment of uraemia. Clin. Nephro., 11,81-83.

Sparks, R.E., Saleman, R.M., Meier, P.M., Litt, M.H., and Lindon, O. (1972). Binders to remove metabolite from the G I tract. Trans. Am. Soc. Artifi. Int. Organs. XVIII, 458-464.

Taillandier, P., Cazottes, M. L., and Strehaiano, P. (1994). Deacidification of grape musts by Schizosaccharomyces entrapped in alginate beads : a continuous-fluidized-bed process. The Chem. Eng. Journal, 55, B29-B33.

Walker, J.M., Jacobson, R.L., Stephen, W.J., and Rose, D. (1977). The role of adsorbents in the wearable artificial kidney. pp. 137-149. In : Gilchrit, T. (ed.), Artificial Organs. Macmillan Press, London.

Wong, H. and Chang, T.M.S. (1991). The microencapsulation of cells within alginate poly-L-lysine microcapsules prepared with the standard single drop technique: histologically identified membrane imperfections and associated graft rejection. Biomat. Artfi. Cells and Immob. Biotechnol., 19(4), 687-697.

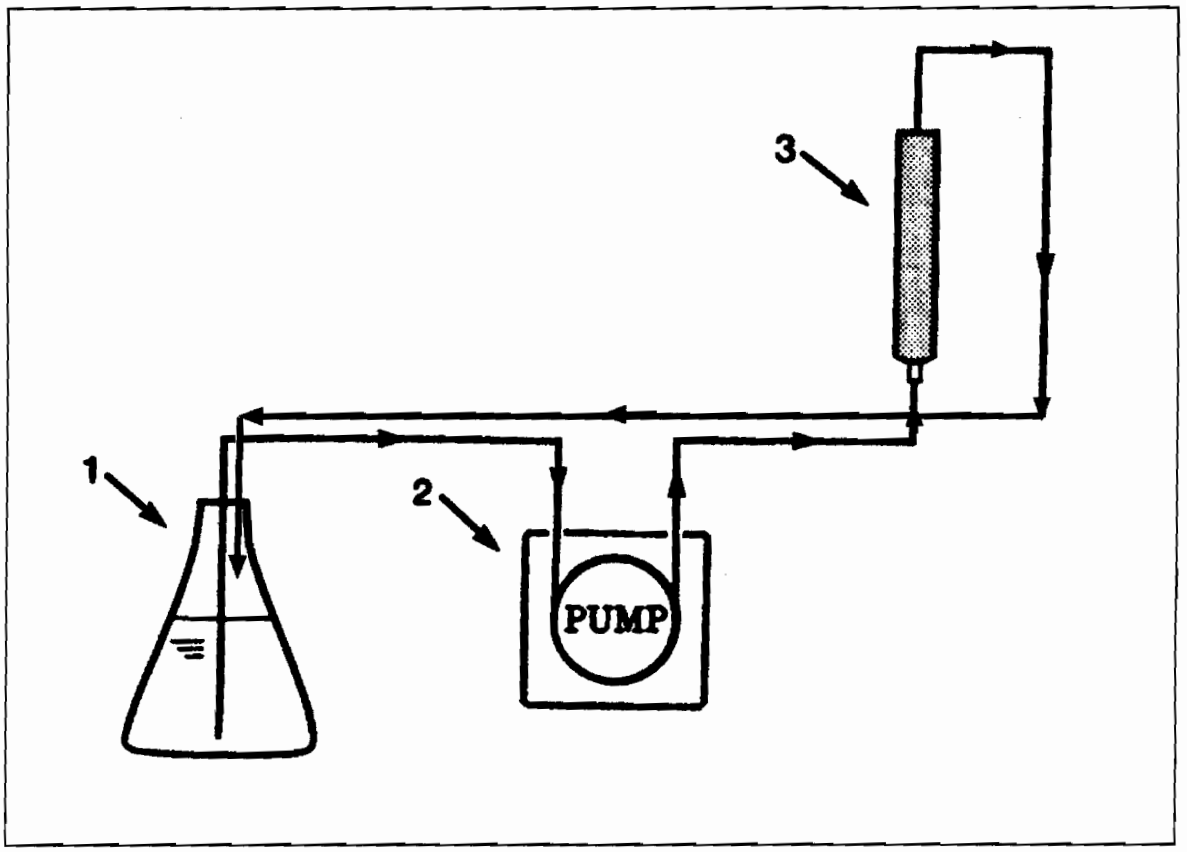


Figure 4.1: A schematic diagram of the apparatus set-up and column bioreactor used for plasma urea and ammonia removal study. 1. Reservoir of substrate solution, 2. Peristaltic pump, 3. Column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH5 cells.

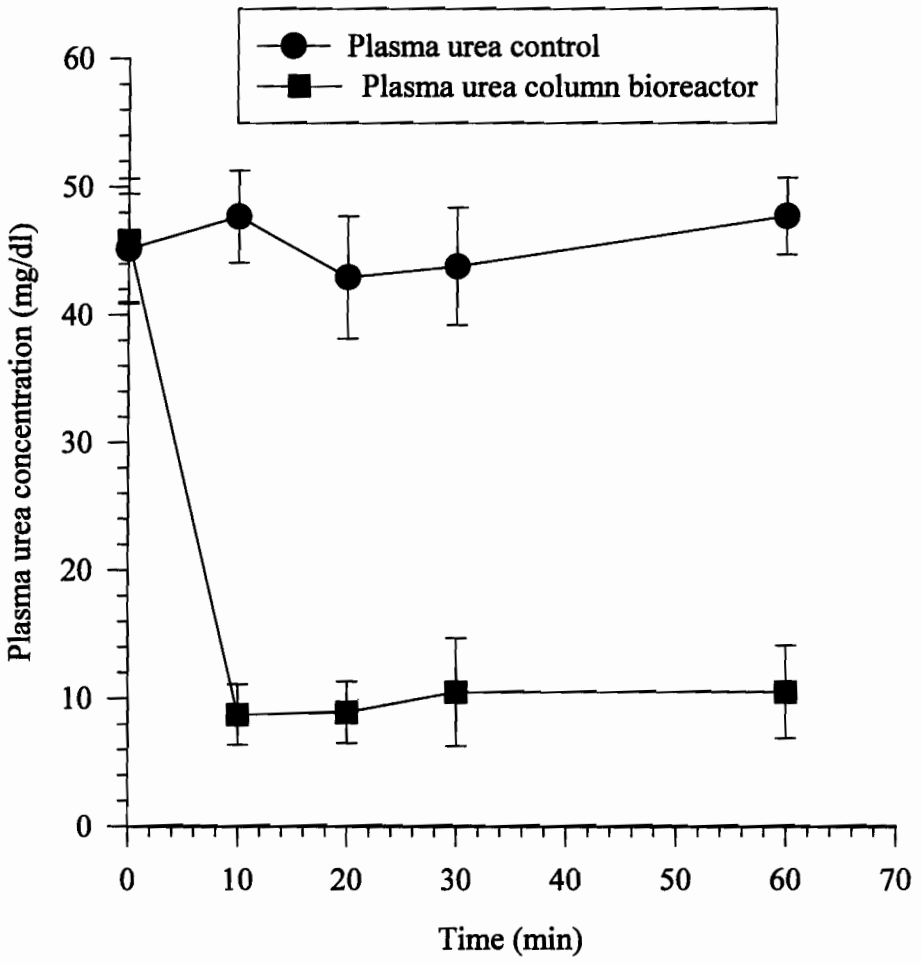


Figure 4.2: Plasma urea removal profile of column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH 5 cells. Operating conditions : T = 25 ^{0} C, column flow rate (F) = 35.40 ml/hr, Biomass used = 5.0 mg ,and Total volume of plasma used (V_L) = 5.0 ml.

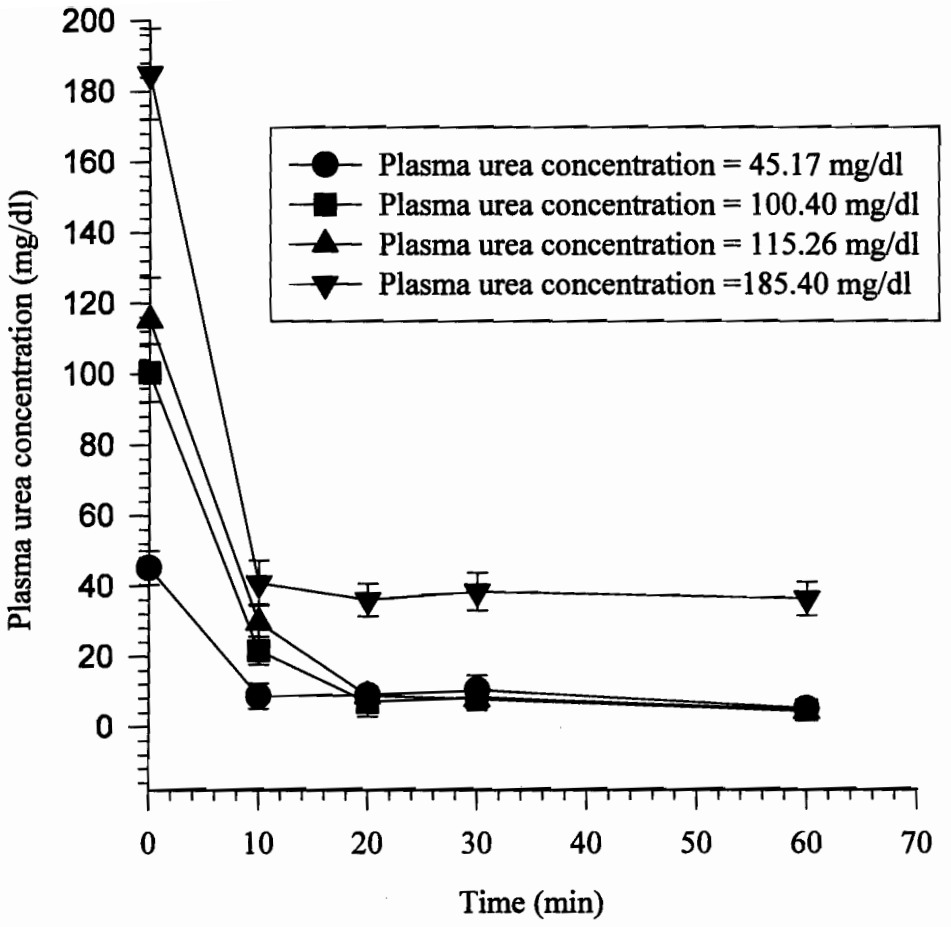


Figure 4.3: Performance of column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH 5 cells as function of initial plasma urea concentrations. Operating conditions : T = 25 ^{0} C, column flow rate (F) = 35.40 ml/hr, Biomass used = 5.0 mg, and Total volume of plasma used (V_L) = 5.0 ml.

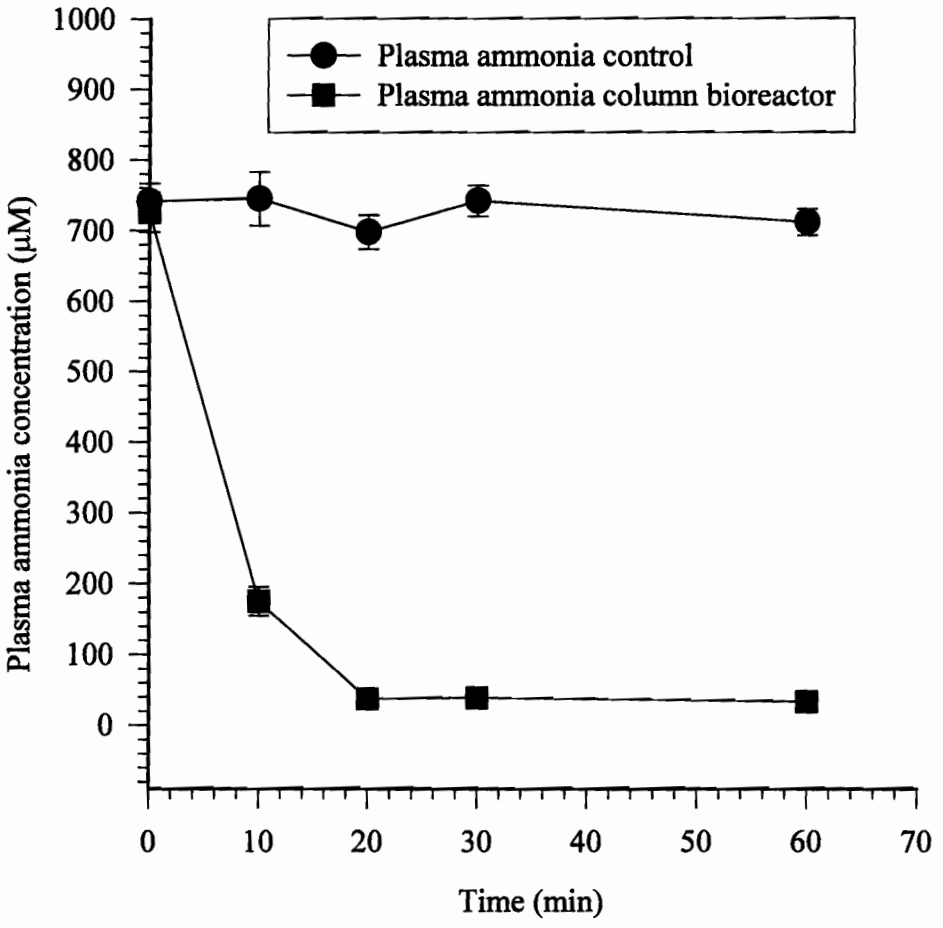


Figure 4.4: Plasma ammonia removal profile of column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH 5 cells. Operating conditions : T = 25 ^{0} C, column flow rate (F) = 35.40 ml/hr, Biomass used = 5.0 mg, and Total volume of plasma used (V_L) = 5.0 ml.

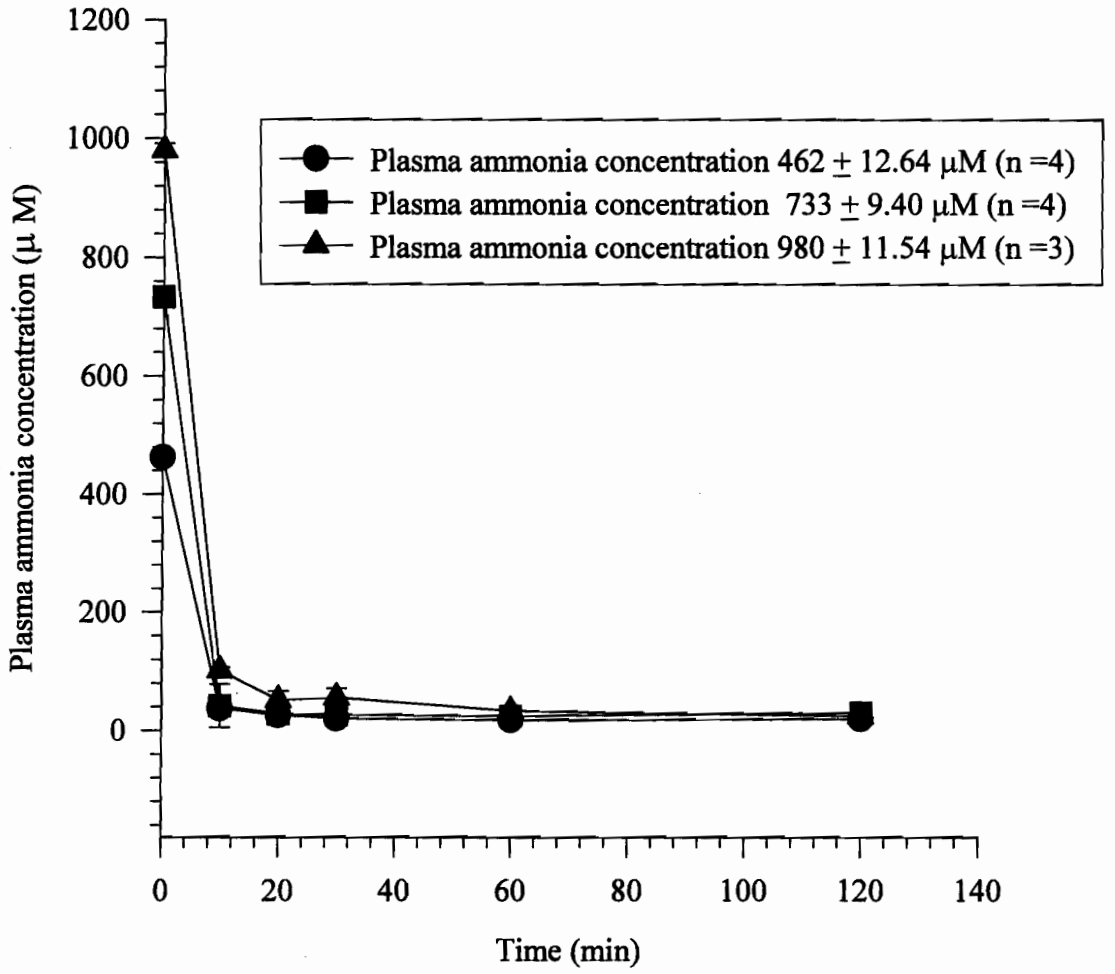


Figure 4.5.: Performance of column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH 5 cells as a function of initial plasma ammonia concentrations. Operating conditions : T = 25 ^{0} C, Column flow rate (F) = 35.40 ml/hr, Biomass used = 5.0 mg, Total volume of plasma used (V_L) = 5.0 ml.

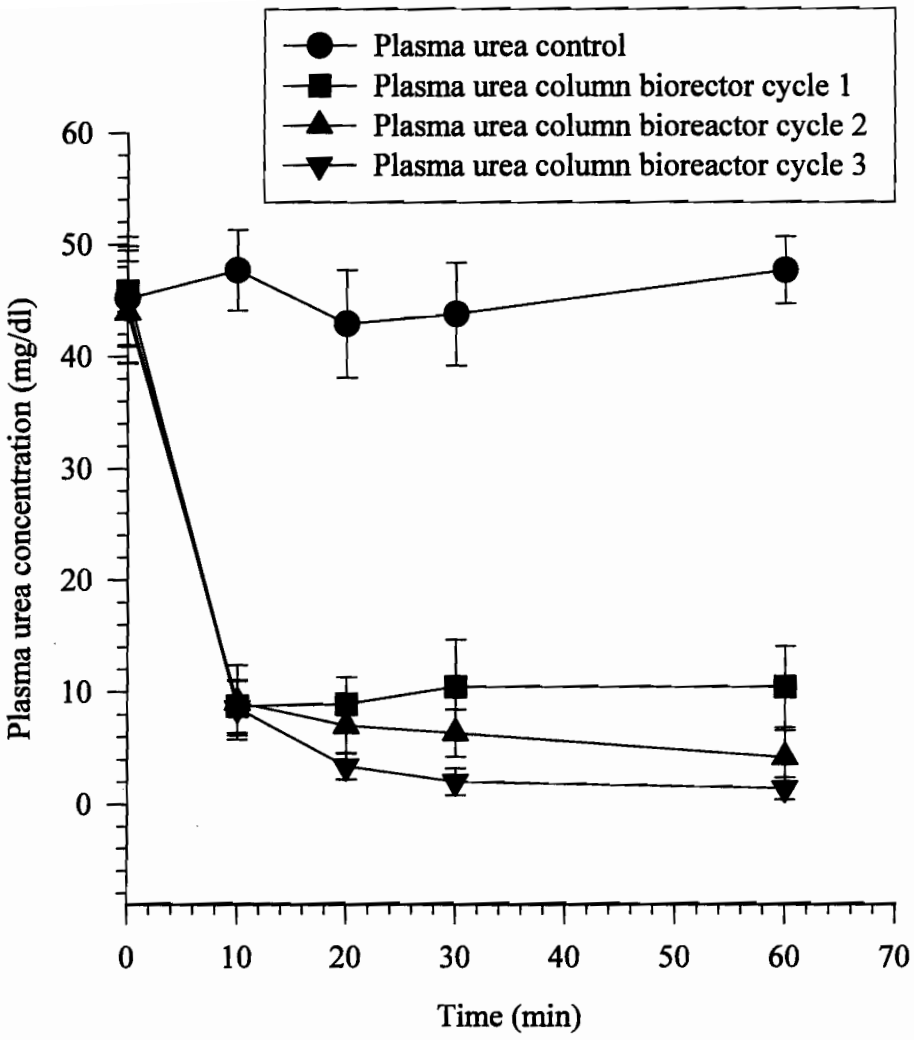


Figure 4.6: Operational stability of column bioreactor containing microencapsulated genetically engineered bacteria *E. coli* DH 5 cells, in terms of plasma urea removal efficiency. Operating conditions : T = 25 ^{0} C, column flow rate (F) = 35.40 ml/hr, Biomass used = 5.0 mg, and Total volume of plasma used (V_L) = 5.0 ml..

CHAPTER 5

A New Approach using Artificial Cells containing Genetically Engineered *E. coli* DH 5 Cells for Urea and Ammonia Removal

S. Prakash and T.M.S. Chang

Submitted, ASAIO Journal, Dec. 1995.

5.1 ABSTRACT

We have introduced a new approach using artificial cells containing genetically engineered microorganisms for the removal of urea and ammonia. The potential use of this new approach for urea and ammonia removal from the body fluid compartments in kidney failure, liver failure, and other diseases is specifically addressed. Compared to standard approaches, using this new concept, the in-vivo urea removal rate was 30 times higher than oxystarch and 95 times higher than urease-zirconium phosphate.

5.2 INTRODUCTION

The search for suitable means of urea and ammonia removal has resulted in several approaches (1-3). These can be broadly divided into five groups: i) Biological approaches: organ transplantation, etc., (1-3) ii) Physical approach: use of hemoperfusion, dialysis, etc. (1-7), iii) Chemical approach: this includes the use of oral ingestion of adsorbents that binds urea and ammonia in the intestine, e.g. oxystarch, zirconium phosphate etc.(7-13), and iv) Enzymatic approach: one example is the use of micoencapsulated urease that convert urea into ammonia which is then removed by adsorbent (4-6,14-8). The other example is the use of microencapsulated multienzyme systems that convert urea and ammonia into essential amino acids (19-20), and v) Colonisation of the intestinal tract with microorganisms which can remove urea and ammonia (21).

Dialysis machines are complex (1-3). Coated charcoal hemperfusion does not remove urea or ammonia (6,7). High dosages are required for oxystarch and zirconium phosphate (8,11,17). The conversion rate of the multienzyme complex are still low at present (19,20). Colonisation of the intestine with microorganisms did not become clinically

practical (21). Therefore, it is essential to look for a new approach for urea and ammonia removal.

The exploding advancement in molecular biology has furnished a variety of genetically engineered cells with many specialised functions. Recombinant DNA technology can genetically engineer cells and bacteria to perform specialised functions which otherwise are not possible (22). This can be combined with artificial cells biotechnology (23-26) to provide a specialised environment to the encapsulated materials. This study is to exploit and combine the use of these two powerful technologies, the recombinant DNA technology and artificial cells. In this article we report the use of microencapsulated genetically engineered bacteria *E. coil* DH5 cells for the removal of urea and ammonia.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals

Alginic acid (low viscosity, Lot 611994) and poly-L-lysine (MW 16,100, Lot 11H5516) were purchased from Kelco and Sigma Chemical Co. (St. Louis, MO, USA) respectively. Unless specified, chemicals were obtained commercially and not further purified before use and they were of analytical reagent grade.

5.3.2 Microorganism and Culture Conditions

Genetically engineered bacteria *Escherestia coli* DH5, containing the urease gene from *Klebsiella aerogens*, was a generous gift from Prof. R. P. Haussinger (27,28). Luria-Bertani (LB) growth medium was used for primary cell cultivation (22). The composition of LB medium was of 10.00 g/L bactotryptone (Difco), 5.00 g/L bacto yeast extract (Difco),

and 10.00 g/L sodium chloride (Sigma). The pH was adjusted to 7.5 by adding about 1.00 ml of 1.00 N NaOH. Media were then sterilized in Castle Labclaves for 30 minutes at 250 ^{0} C. Incubation was carried out in 5.00 ml LB in 16.00 ml culture tubes at 37 ^{0} C in an orbital shaker at 120 rpm. For the large-scale production of biomass, for microencapsulation purpose, 250 ml Erlenmeyer flask containing 100 ml LB medium was used.

5.3.3 Microencapsulation Procedure

The details of the microencapsulation procedure are described elsewhere (39). Very briefly, microcapsules containing bacterium *E. coli* DH5 cells were prepared as follows: Log phase bacterial cells were suspended in an autoclaved sodium alginate in 0.9 % sodium chloride solution. The viscous alginate-bacterial suspension was pressed through a 23 gauge needle using a syringe pump (Compact Infusion Pump Model 975, Harvard App. Co. MA). Compressed air through a 16 gauge needle was used to shear the droplets coming out of the tip of the 23 gauge needle. The droplets were allowed to gel for 15 minutes in a gently stirred ice-cold solution of calcium chloride (1.4 %). After gelation in the calcium chloride, alginate gel beads were coated with polylysine (0.05 % in HEPES buffer saline, pH 7.20) for 10 minutes. The beads were then washed with HEPES and coated with an alginate solution (0.1 %) for 4.00 minutes. The alginate-poly-L-lysine-alginate capsules were then washed in a 3.00 % citrate bath (3.00 % in 1:1 HEPES-buffer saline, pH 7.20) to liquefy the gel in the microcapsules. The microcapsules were called APA microcapsules and were stored at 4 ^{0} C and used for experiments.

5.3.4 Microcapsule Storage Condition

After microencapsulation the microcapsules were washed properly several times (two to three times) with cold sterile water. The microcapsules were resuspended in the Aggrobacteriium minimum broth (AG minimal media) at 4-10 ^{0} C (30). This media, unlike L. B. media, does not support the growth of *E. coli*, it has however all the components

which is necessary to maintain biochemical activity (30). Before the use microcapsules were washed in normal saline and used for the experiment.

5.3.5 Reaction Media for In-Vitro Urea and Ammonia Removal Experiments

Reaction media in all experiments consisted of 1.00 g/L glucose, 20.00 mg/L magnesium sulphate, 30.00 mg/L dipotassium monohydrogen phosphate, and 0.07 mg/L vitamin B_{12}. As required, filter sterilized urea was added in the reaction media to make the urea concentration 100 mg/dl. Plasma from a bovine source was used for the plasma urea and ammonia removal experiments and filter-sterilized urea was added to the plasma. This was done to mimic the uremic patient's plasma characteristics in-vitro.

5.3.6 Experimental Procedure for the In-Vitro Studies

The bacteria were grown in L B medium. Log phase bacterial cells were harvested by centrifuging at 10,000 g for 20 min. at 4 ^{0}C. The cell mass was then washed five times with sterile cold water to remove media components. Cells were then weighed and used for the urea and ammonia removal studies by free bacteria. For the microencapsulated urea and ammonia removal studies the equivalent masses of the cells were microencapsulated and used. In all in-vitro studies, reactions were performed in 250-ml Erlenmeyer flasks at 30 ^{0}C and 100 rpm, unless otherwise mentioned. The Lab-Line orbital environ-shaker equipped with thermal control and air quality were used for this purpose. Sampling was carried out aseptically at designated times. Bacterial cells, in the free bacteria removal studies, were removed from the sample by centrifugation at 15,000 rpm and the supernatant analysed. For the plasma urea and ammonia removal studies we used bovine plasma with filter-sterilized urea added to a final urea level comparable with that of kidney failure patients (24). In all the experiments, the ratio of the reactor volume to the amount of microencapsulated bacteria used was held constant.

5.3.7 In-Vivo Studies in Uremic Rats

For in-vivo animal studies, microcapsules containing 5 mg of log phase genetically bacteria were first suspended in 0.8-1.0 ml sterile normal saline in a 5 ml syringe. The floating microcapsules were then force fed orally using curved 12G-3 1/2 stainless steel needles. Sampling was done by taking blood samples from the rat tail after sedating using appropriate amounts of anaesthetic drugs that had been reported for not having any side effects on renal or hepatic functions. The drugs used were atravet (acepromazine) and ketaset (ketamine) in a concentration of 75 mg / kg and 5-10 mg / 9 kg intramuscularly, respectively. Blood samples were taken using a small 23 G1 precision Glide needle and 10 CC syringe. Blood samples were centrifuged immediately using an eppendroff centrifuge and plasma was collected and analysed for urea and ammonia.

5.3.8 Urea and Ammonia Determination

Urea concentrations were determined based on quantitative measurements of blood using the BUN kit purchased from Sigma Chemical Co. USA (31). Ammonia was analysed using a fluorescent light scattering Multistat III microcentrifugal analyser. This was based on the reductive amination of 2-oxoglutarate, using glutarate dehydrogenase (GLDH), and reduced nicotinamide adenine dinucleotide (NADH). The decrease in absorbance at 340 nm due to the oxidation of NADH is proportional to ammonia concentration.

5.4 RESULTS

The specific goal of this study is to explore the potential of encapsulated genetically engineered bacterial cells for the removal of urea and ammonia in kidney failure, liver

failure and other diseases. This will also serve as a feasibility study for our broader goal to explore the use of this for other clinical conditions.

The alginate-poly-L-lysine-alginate (APA) microcapsules containing genetically engineered *E. coli* DH5 cells were prepared. The details of the process parameters of preparation of microcapsules and the kinetics of the in-vitro studies are described elsewhere (26,29,32). In the in-vitro studies (Figure 5.1) the microencapsulated bacteria were able to lower plasma urea from 50 $\pm$ 10.12 mg/dl to 28.64 $\pm$ 14.46 mg/dl, 8.64 $\pm$ 4.8 mg/dl, and 6.24 $\pm$ 3.45 mg/dl in 10, 20, and 30 minutes respectively.

An experiment was also designed to explore the possibility of using microencapsulated genetically engineered bacterial cells for ammonia removal in liver failure and other diseases. For this plasma, with an ammonia level of 942 $\pm$ 82 μM/L, was used. Microencapsulated bacteria were able to deplete plasma ammonia at a very high rate (Figure 5.2). When we compare this in-vitro urea removal with the existing adsorbents (Figure 5.3), we found that microencapsulated genetically engineered bacterial system much higher. A very small dose is required for the microencapsulated bacteria compared to adsorbents commonly used urea removals (Figure 5.3).

Based on these and our other in-vitro experimental results (26,29,31), we have carried out following the preliminary in-vivo experiments. We used surgically induced uremic rat models. A daily feeding of 5 mg encapsulated bacteria to the 300-325 g body weight uremic rats resulted in a decrease of plasma urea from 52.08 $\pm$ 2.37 mg % to 37.53 $\pm$ 3.32 mg % within the first 48 hours. By the end of 3rd day, plasma urea level was only 12.46 $\pm$ 3.16 mg %. This was maintained throughout the treatment period of up to 24 days.

Using the results obtained in our preliminary in-vivo studies we evaluated the total in-vivo urea binding capacity per milligrams of bioreactant. Figure 5.4 summarises the comparison of this new approach of urea removal using APA encapsulated bacteria with the presently used counterpart oral adsorbents. Results show that APA encapsulated bacteria were able to remove 3.20 $\pm$ 0.67 mg of urea / mg of biomass on the day two and 8.00 $\pm$ 1.32 mg / mg of biomass on day four whereas, oxystarch and Urease-zirconium-phosphate at their best can remove 0.103 mg of urea / mg oxystarch and 0.033 mg of urea / mg of urease-zirconium-phosphate only . This approach, therefore, has the ability to lower plasma urea and ammonia better than the presently used system. The details of the kinetics and procedure of in-vivo results will follow.

5.5 DISCUSSION

The existing methodologies for the treatment of uremia are very complex and expensive (41,4,5,6,8,11,34,35). A recent review warns that the world would not be able to afford uremia therapy in the 21st century (2). The requirement of a new cost effective technology is, therefore, urgent. This will also allow the developing countries to face the challenge of uremia treatment.

The main objective of our present study is to explore the potential of two technologies, recombinant DNA technology and artificial cells, for potential clinical applications. The specific objective of this research is to find a new approach to manage urea and ammonia levels in kidney failure, liver failure and other diseases. Accordingly, we encapsulated the genetically engineered *E. coli* DH5 cells in microcapsules. The hypothesis of using bacteria to treat human diseases was suggested by Metchnikoff as early as in 1908 (37). However, very little attention has been given to the above hypothesis and only few

published reports are available such as the use of bacteria by Setala for uremia (21) and recently by Sarvedra for preventing diarrhoea in infants (36). For the first time we report that the APA encapsulated genetically engineered bacteria *E. coli* is capable of plasma urea and ammonia removal both in in-vitro and in-vivo. Our present study supports the finding of our previous in-vitro studies in the efficiency of artificial cells encapsulated genetically engineered bacteria *E. coli* DH5 cells (26,29,32,33). Using a single pool model in these earlier in-vitro studies, we found that encapsulated bacteria can remove urea with a much greater efficiency than generally used oxystarch and urease-zirconium phosphate. The total urea removed is 3.20 ± 0.67 mg urea / mg of biomass on the day two and 8.00 ± 1.39 mg / mg of biomass on day four. Oxystarch and urease-zirconium-phosphate on the other hand have the capacity to remove 0.103 mg of urea / mg oxystarch and 0.033 mg of urea / mg of urease-zirconium-phosphate only (7-9,11-14, 16, 26,29,32). Further, current oxystarch and urease-z.p. system work under specialised conditions which is not necessarily available during the actual treatment. For example, oxystarch has good capacity of urea and ammonia binding at a specific pH only (12,16).

Efficient removal of ammonia from the body fluid compartment is also required in a number of conditions including liver failure (1,6,11,14,19,20,12). In this study (Figure 2) we have shown that efficient ammonia removal is possible by using microencapsulated genetically engineered bacteria. Plasma ammonia concentration of uremic rats fell from 560 $\pm$ 51 μM/L to 144 $\pm$ 24.70 μM/L during the urea removal experiment. This is a decrease of about 72 %. This observed efficiency of ammonia removal of encapsulated bacterial systems higher than the commercially available Redy machines (13).

This study demonstrates the feasibility of oral feeding of microencapsulated genetically engineered microorganisms in general and genetically engineered bacteria in particular for the disease management. This study also provides the foundation for the further development of oral microcapsules containing genetically engineered for urea and ammonia removals in kidney failure, liver failure and other diseases.

5.6 CONCLUSIONS

Microencapsulated genetically engineered *E. coli* DH5 cells have high in-vitro efficiency in lowering plasma urea and ammonia. In-vivo results show that microencapsulated genetically engineered bacterial cells can efficiently lower systemic urea in uremic rats. Encapsulated bacteria were able to lower plasma urea level from 52.08 mg % to 37.52 mg % within the first 48 hours and a normal plasma urea level was achieved for a period of 24 days using this treatment approach. Under similar conditions, the same bacteria can lower plasma ammonia from 519 ± 51.26 μM to 144.00 ± 24.70 μM during the first 48 hours of the treatment. Our studies show that this novel approach is several hundred fold more efficient in removing urea and ammonia than the standard oxystarch and urease-zirconium phosphate.

5.7 ACKNOWLEDGENTS

T.M.S.C. acknowledges the research support of the Medical Research Council of Canada and Quebec Ministry of Higher Education Science and Technology "Centre for Excellence in Biotechnology Virage". S.P. acknowledges a National Overseas Scholarship from the government of India. The generous gift of the organism used in this study is from Prof. R P. Haussinger.

5.8 REFERENCES

1. National kidney and urological diseases clearhous, 3 Information Way, Bethesda.

NIH Publication No. 95-3925 .1995.

2. Friedman, E.A.: Facing the reality: The world can not afford uremia therapy at the start of 21 century . Artificial Organs, 19(5) ,481-485.1995.

3. Drukker, W., Parsons, F. M., and Mahar, J. F.: Replacement of renal functions by Dialysis. Martinus Nijhoff, Boston. Artif. Int. Org. 27: 24-30.1983.

4. Chang, T. M. S.: Semipermeable aqueous microcapsules (artificial cells) with emphasis on experiments in extracorporeal shunt system. Trans. Am. Soc. Artif. Int. Org. 12: 13-19 .1966.

5. Chang, T. M. S.: Microencapsulated artificial kidney : including updated preparative procedures and properties. Kidney Int. 10: S218-224.1976.

6. Chang, T. M. S.: Hemoperfusion alone and in series with ultra centrifugation or dialysis for uremia, poisoning, and liver failure. Kidney Int. 10: S305-311.1976.

7. Friedman, E. A., Fastcook, J., Beyer, M. M., Rattazzi, T., and Josephson, A. S. Combined oxystarch-charcoal trial in uremia, sorbent induced reduction in serum cholesterol. Kidney Intern. 13, S273-S276.1976.

8. Walker, J. M., Jacobson, R. L., Stephen, W. J., and Rose, D.: The role of adsorbents in the wearable artificial kidney. In: Gilchrist, T. (ed.), Artificial Org. Macmillan, London. Willey, New York. pp. 521-528.1977.

9. Friedman, E. A. Rationing of uremia therapy. Artif. Org., 16(1):90-97.1992.

10. Steven Z., and Friedman, E. A. In: sorbent and their clinical applications (Carmelo G. ed.), Potential of oral sorbent treatment of uremia. 275-294 .1980.

11. Kjellstrand, G., Borger, H. Pru, C. G. and Fink, D.: On clinical use of microencapsulated zirconium phosphate-urease for the treatment of chronic uremia. Hemoperfusion and Artificial Organs (T.M.S. Chang, ed.).206-216 .1983.

12. Sparks, R. E., Saleman, R. M., Meier, P. M., Litt, M. H., and Lindon, O.: Binders to remove metabolite from the GI tract. Trans. Am. Soc. Artif. Int. Org. 18: 458-464.1972.

13. Gardner, D.L., Emerling, D.C.,Williamson, K.D., Baytos, W.C., and Hassler, C.R.

Encapsulated adsorbents for removal of nitrogenous metabolites via oral ingestion. Kidney Int. 7, S393.1975.

14. Chang, T.M.S.: Artificial cells with emphasis on bioencapsulation in biotechnology. Biotechnology Annual Rev. 1: 267-296. 1995.

15. Cattaneo, M., Chang, T. M. S.: The potential of microencapsulated urease-zeolite oral sorbent for the removal of urea in uremia. J. Am. Soc. Artif. Int. Org. 37: 80-87.1991.

16. Esposito, R., Carmelo, G.: Polyhalides: In: G. Caramelo (ed.), Sorbents and their clinical applications. Academic press, London. pp.133-153 .1980.

17. Chang, T. M. S.: Biotechnology of artificial cells including application to artificial organs, p.p. 53-73. In: M. Moo Young (ed.), Comprehensive biotechnology: The principle applications & regulation of biotechnology in industry, agriculture, and medicine. Pergamon press, New York .1985.

18. Chang, T. M. S.: Biotechnological and medical application of immobilization of hepatocytes, microorganisms and enzyme systems by microencapsulation in artificial cells. Enz. Eng. 10: 331-339 .1990.

19. Gu, K. F., and Chang, T. M. S.: Conversion of alpha-ketoglutarate into L-glutamic acid with urea as ammonia source using multienzyme system and NAD immobilized by microencapsulation with artificial cell in bioreactor. Biotechnol. Bioeng. 32: 363-368.1988.

20. Gu, K. F. and Chang, T. M. S.: Conversion of urea or ammonia in to essential amino acids, L-leucine, L-valine and L-isoleucine using artificial cells containing an immobilized multienzyme system and dextran-NAD. Yeast alcohol dehydrogenase for enzyme recycling. J. Biotech. Applied Biochem. 12: 227-236.1991.

21. Setala, K., Heinonen, H., and Schreck-Purla: Uremic waste recovery. Proc. Conf. EDTA, 9: 514-520. 1972.

22. Sambrook, J., Fritsch, E. F., and Maniatis, T.: Molecular cloning : a laboratory

manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York,1989.

23. Chang, T. M. S.: Semipermeable microcapsules. Science, 146: 524-525.1964.

24. Chang, T. M. S.: Methods in the medical application of immobilized proteins, enzymes and cells. Methods in Enzymology, 137: pp. 444-457.1988.

25. Chang, T. M .S.: Living cells and microorganisms immobilized by microencapsulation inside artificial cells, pp. 183-194. In: M. F. Goosen (ed.), Fundamentals of animal cell encapsulation and immobilization. CRC press, Boca Raton, FL .1993.

26. Chang, T. M. S. and Prakash, S.: Artificial cells and genetically engineered microencapsulated *E. coli* cells, for urea and ammonia removal: "Methods in Molecular Biology" Series, Humana Press, USA Chapter 75 (in press) 1995.

27. Mobley, H. L. and Haussinger, R. P.: Microbial ureases: Significance, regulation, and molecular characterization. Microbiol. Rev. 53: 85-108.1989.

28. Scoot, B., Mulrooney, H. Stuart, P., and Haussinger, R. P.: Regulation of gene expression and cellular localization of cloned *K. aerogens* urease. J. Gen. Microbiol. 135: 1769-1776.1989.

29. Prakash, S. and Chang, T. M. S.: Preparation and in vitro analysis of genetically engineered *E. coli* DH5 cells, microencapsulated in artificial cells for urea and ammonia removal : Biotechnology and Bioengineering, Vol.46, p.p. 621-626,1995.

30. Marincs, F. and White, D.W.R.: Immobilization of *E. coli* expressing the lux gene of Xenorhabdus luminescence; Applied and Environmental Microbiology, Oct, p.p. 3862-3863.1994.

31. Symmour, A. C., Philip, P. C.: Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem. 209: 145.1954.

32. Prakash, S. and Chang, T. M .S.: Microencapsulated genetically engineered *E. coli* cells containing genes form *K. aerogens* for urea and ammonia removal. Biomat. Artifi. Cells Immob. Biotechnol. 19: 687-697.1993.

33. Prakash, S and Chang, T. M .S.: Microencapsulated genetically engineered *E. coli* cells for urea and ammonia removal in kidney failure and liver failure: IEEE transaction in Biomedical engineering, (in press). 1995.

34. Cohen, B. D.: Uremic toxins. In : Uremia, an international conference on pathogenesis, diagnosis and therapy. pp. 1-11.1972.

35. Kolff, W. J.: Artificial kidney and artificial heart: Future considerations. Int. J. Artif. Org. 13: 404-406. 1990.

36. Saarvedra M. J., Baumann N., Oung, I., Perman, J. and Yolken R.H.: Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhea and shedding of rotavirus. Lancet, 344, pp. 1046-1049. 1995.

37. Tasovatz, B. L'entero-colite aigue bacterienne chez le nourrssion essai de traitement par le bacille bifidus. Ann. de Pediatrie. 22:291-297.1964.

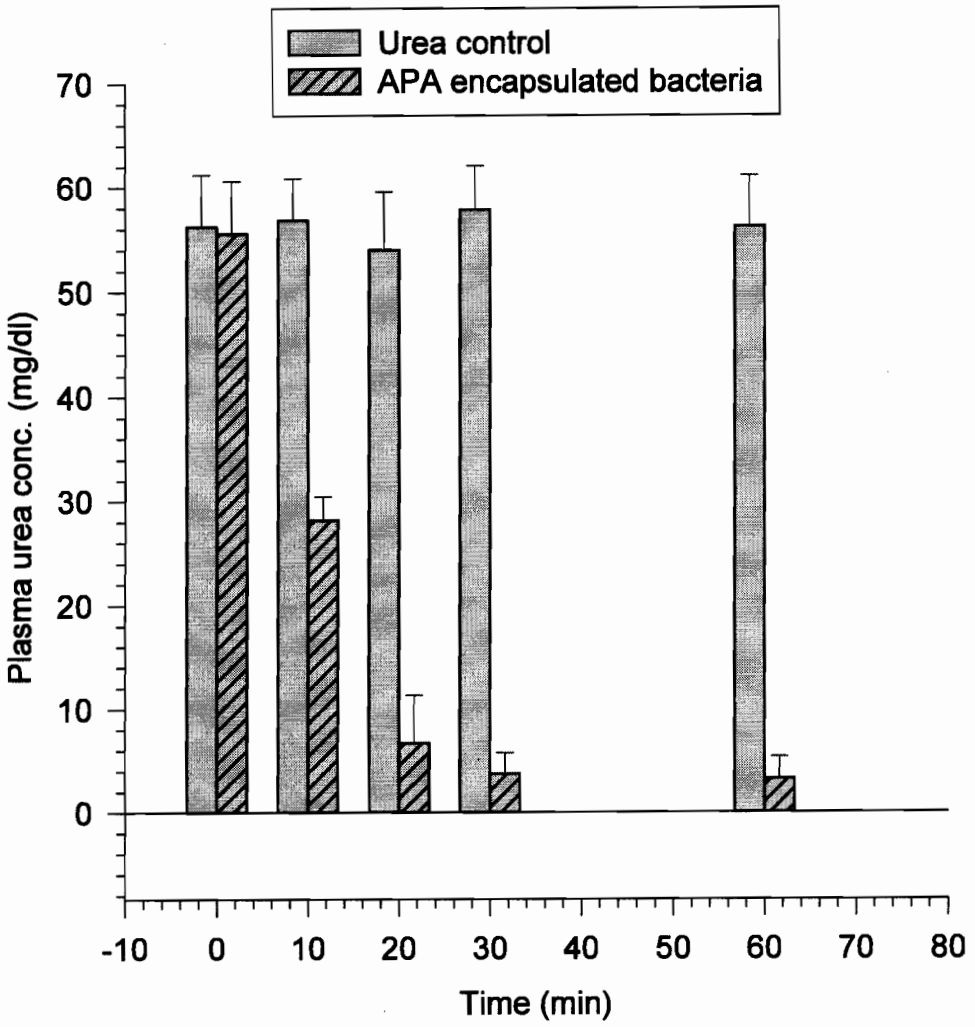


Figure 5.1: In-vitro plasma urea depletion profile of microencapsulated genetically engineered bacteria *E. coli* DH5 cells (n =12, P = 0.0021).

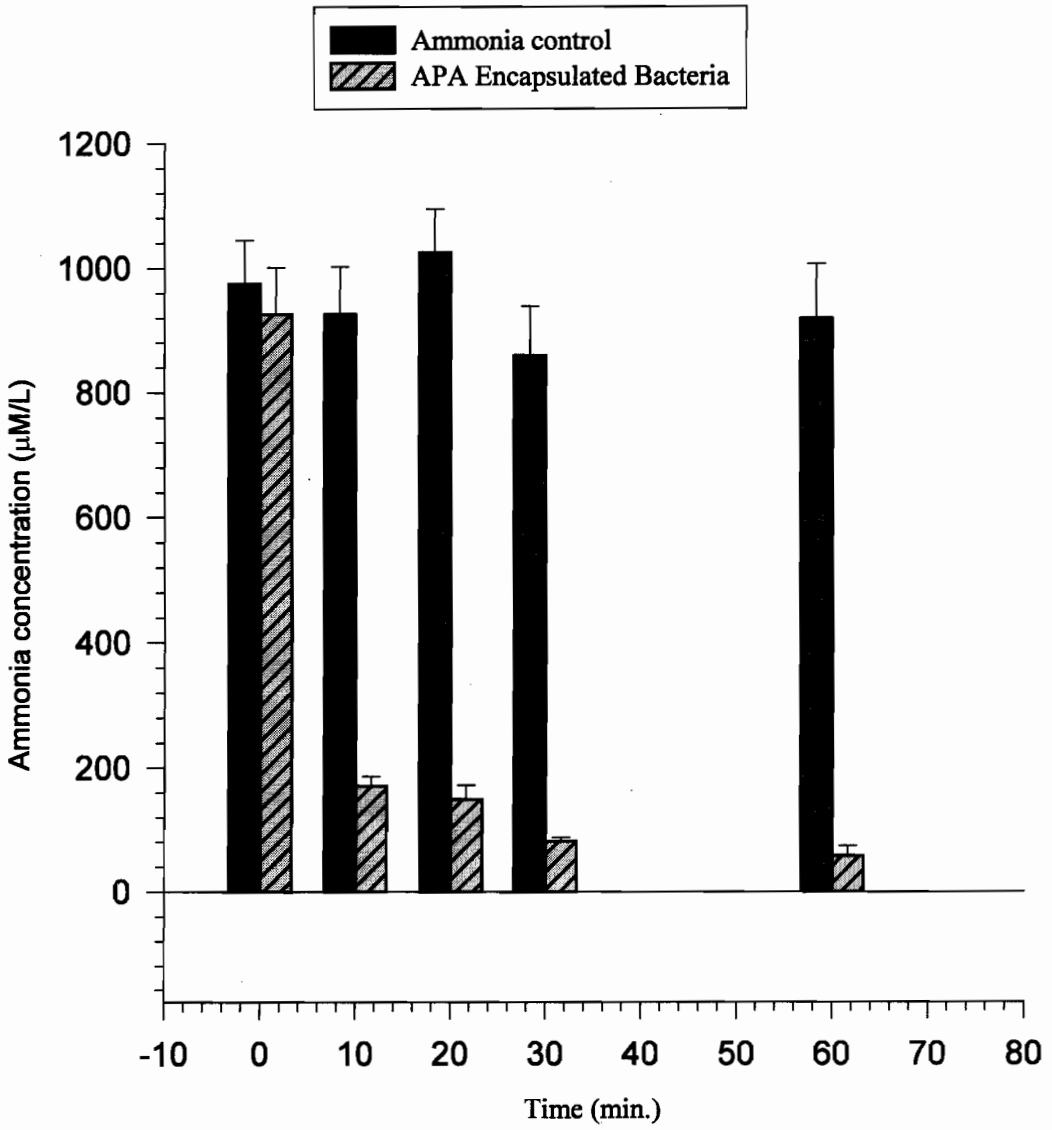


Figure 5.2: In-vitro plasma ammonia depletion profile of microencapsulated genetically engineered bacteria *E. coli* DH5 cells (n = 12, P = 0.018).

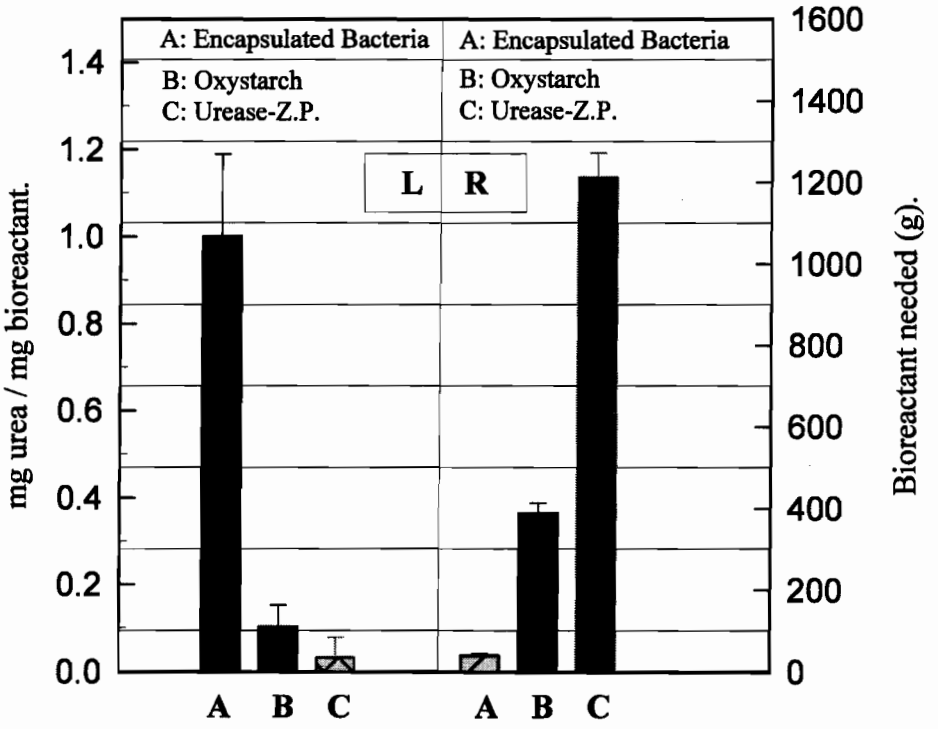


Figure 5.3: L) Comparative study of urea removal efficiency of microencapsulated genetically engineered bacteria in-vitro performance (n =16, P = 0.032 student-t test two tailed paired analysis) and in-vitro available data for urea removal by oxystarch and urease-zirconium phosphate. R) Amount of bioreactant needed to lower urea concentration from 100 mg/dl to 8.76 mg/dl in a 70 kg adult uremic man.

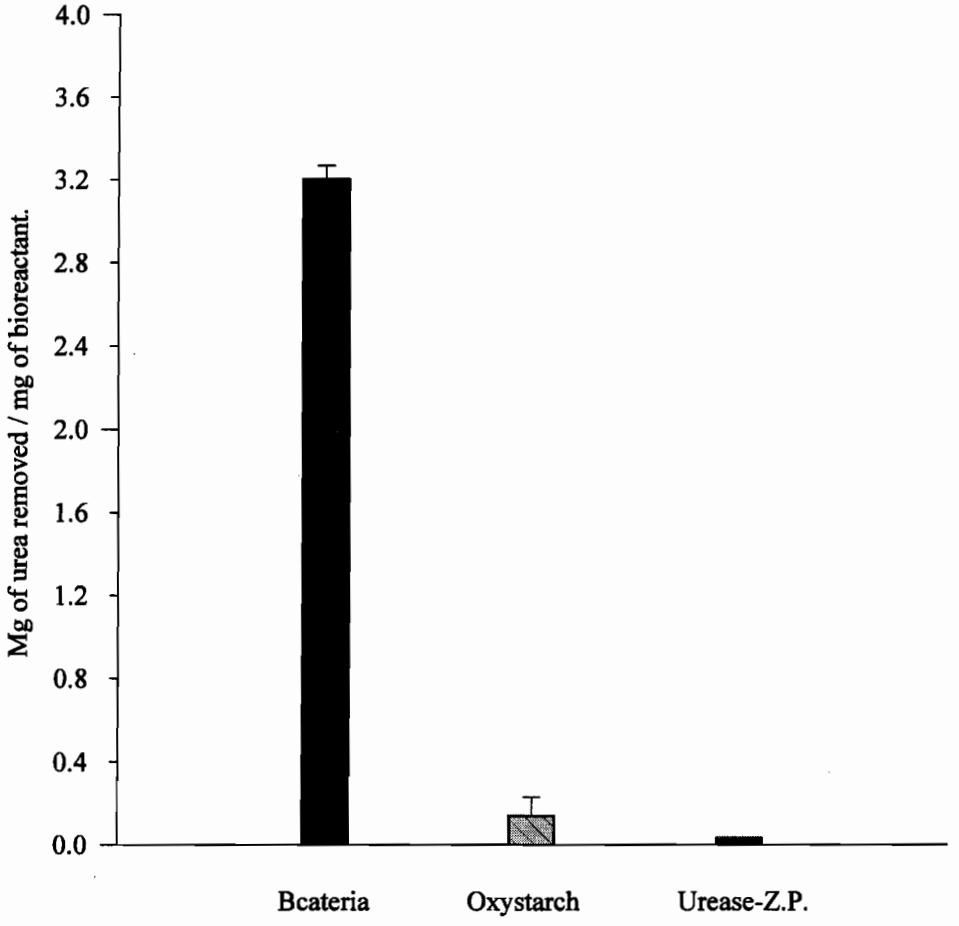


Figure 5.4: Comparative study of urea removal efficiency of microencapsulated genetically engineered bacteria in-vivo performance on uremic rats (n =12, P = 0.0021 student-t test two tailed paired analysis) and in-vitro available data for urea removal by oxystarch and urease-zirconium phosphate.

CHAPTER 6

Microencapsulated Genetically Engineered live *E. coli* DH5 Cells Administered Orally to Maintain Normal Plasma Urea Level in Uremic Rats

S. Prakash and T.M.S. Chang

Submitted to Nature, Feb. 1996.

6.1 ABSTRACT

This article describes a new therapeutic approach of using genetically engineered bacterial cells. We use the artificial cell concept [1-3] to allow oral administration of genetically engineered bacterial cells. In this study, microencapsulated genetically engineered bacteria *E. coli* DH5 cells for the removal of urea is used as an example and as a feasibility study. We orally administered daily 11.15 $\pm$ 2.25 mg / kg of body weight of microencapsulated genetically engineered bacteria *E. coli* DH5 cells to uremic rats. The plasma urea concentration during the control period was as high as 52.08 $\pm$ 2.06 % mg . During the test period the plasma urea levels was maintained in a normal range of 10.02 $\pm$ 2.5 % mg during the entire test of 21 days. On discontinuation of oral administration, the plasma urea returned to uremic level reaching 37.60 $\pm$ 8.2 % mg in three days only. This feasibility study shows the potential possibility of using this approach for many types of microencapsulated genetically engineered cells.

6.2 INTRODUCTION, RESULTS, AND DISCUSSION

The past two decades have experienced an explosive increase in molecular biology research [4,5,6]. As a result, a number of genetically engineered cells, with many special features, have become available. It is difficult to make use of the unique therapeutic properties of these genetically engineered cells since they cannot be injected. This article shows that by using the concept of artificial cells microencapsulated genetically engineered bacteria *E. coli* DH5 cells can be given orally for the removal of urea in uremic rats.

As kidney fails, substances normally excreted in the urine are retained in the blood and body tissues [7,8]. This results in an increased concentration of waste metabolites including urea and presently we do not have an efficient method to remove it [7,8,9,10]. Having

demonstrated that alginate-poly-L-lysine-alginate (APA) membrane encapsulated genetically engineered *E. coli* DH5 cells can effectively remove urea and ammonia from the plasma in-vitro [11,12,13] here we now report the results of our in-vivo studies on experimental uremic rats.

The alginate-poly-L-alginate (APA) membrane microcapsules (Figure 6.1) were prepared as described elsewhere [12]. Throughout the control and treatment periods the rats were fed normal chow. During the treatment, 5 mg of APA encapsulated bacteria were given daily to each uremic rat. For feeding microcapsules were suspended in 0.8-1.0 ml sterile saline in a 5 ml syringe and administered orally using a curved 12 G-3 1/2 gastric lavage tube. Control microcapsules containing no bacteria were fed to normal and uremic rats as positive and negative controls. Rats were sedated with atravet (acepromazine) and ketaset (ketamine) in a concentration of 75 mg / kg and 5-10 mg / 9 kg intramuscularly, respectively. Using a 23 G precision glide needle and a 10 cc syringe, blood samples were collected from the tail vein and centrifuged immediately to obtain plasma. Plasma is then analyzed for urea using the BUN kit [13].

The plasma urea profile of control animal and nepherectomy rat is shown in Figure 6.2. Results show that for treated animals, the plasma urea concentration was reduced from 54.22 ± 6.08 to 37.53 ± 2.56 % mg in 48 hours. As treatment continued, the plasma urea level dropped to 10.58 ± 0.5, and 10.65 ± 1.34 mg % on days seven and nine respectively. By oral administration, we were able to maintain plasma urea concentration of nephrectomy induced uremic rats closer to normal rat plasma urea concentration for a period of 21 days. When oral treatment was discontinued, the urea level slowly increased back to the uremic level (Figure 6.2). During the course of treatment, plasma ammonia concentration was followed closely and found no increase. Using a single pool model [14] and the encountered urea removal efficiency of encapsulated bacteria, we evaluated the requirement of encapsulated bacterial cells for lowering the plasma urea concentration from 100 mg /dl to 10 mg/dl for a 70 kg adult man. Results show a requirement of only 4.2 g / day of encapsulated cells. We conclude that this new treatment approach, to our knowledge, is

better than any other system available for uremia management and has a great potential to be used in future clinical practice.

Before this, several attempts, including the use of oral feeding of oxystarch and urease-zirconium phosphate, have been made to remove these unwanted metabolites [15,16,17,18]. However, in all previous attempts only oxystarch and microencapsulated urease-zirconium-phosphate (UZP) were successful in removing urea and ammonia [15,17,18,19,20]. The required dosage, however, was too large to allow them for use in the routine treatment of patients [20,21,22,23,24].

This study demonstrates the maintenance of normal urea level in uremic rats by oral administration of microencapsulated genetically engineered bacterial cells. This study has provided the foundation for the development of oral microcapsules containing this and other types of genetically engineered cells for the removal of unwanted metabolites.

6.3 ACKNOWLEDGEMENTS

T.M.S.C. gratefully acknowledges awards from the Quebec Ministry of Higher Education Science and Technology "Center for Excellence in Biotechnology Virage" and the support from the Medical Research Council of Canada. S. P. gratefully acknowledges a National Overseas Scholarship from the Government of India.

6.4 REFERENCES

1. Chang, TMS. Science.146, 524-525 (1964).
2. Chang, T.M.S. Methods in Enzymology. 137, 444-457((1988).

3. Chang, T.M.S. Artificial cells. Encyclopaedia of Human Biology. Academic press, NY. 377-383(1991).
4. Carmen, A.M. Am. J of Human Genetics. 50, 245-260 (1992).
5. Griffin , A.M. and Griffin, H.G. In Molecular Biology : current innovations and future trends part 2, Horizon Scientific Press, England (Oct.1995).
6. Mange, Arthur P. In Applied Genetic Engineering; future trends and problems Noyes Publs., Bioscience.34, 642 (Nov. 1984).
7. Charles, S., David, M., and Stacia, M. In :A Deborah (ed.), Human Physiology: and frontiers. Times mirror college publi., St. Louis. 526-527 (1990).
8. Saul G. Massry, In: Uremic Toxins (Severine et al., ed.) Advances in Experimental Medicine and Biol. 223, 1-17 (1986).
9. Friedman, E.A. Artif. Organs. 19(5), 481-485 (1995).
10. Kolff, W.J. Artificial Kidney and Artificial Heart: Future considerations. Int. J. Artif. Org. 13, 404-406 (1990).
11. Prakash, S. and Chang, T.M.S. Biomat. Artifi. Cells Immob. Biotechnol. 19, 687-697 (1993).
12. Prakash, S. and Chang, T.M.S. Biotechnology and Bioengineering. 46,621-626 (1995).
13. Chang, T.M.S. and Prakash, S. In "Methods in Molecular Biology: Expression and Detection of Recombinant Genes" (ed. Rockey S. Tuan) Humana Press, USA. 1995; Chapter 75 (in press).
14. Symmour, A.C., Philip, P.C. J. Biol. Chem. 209,145 (1954).
15. Sargent, J.A. Kidney Int. 23,S19-25(1983).
16. Esposito, R., Carmelo, G., and G. Polyhalides. In: G. Caramelo (ed.), Sorbents and their clinical applications. Academic press, London..133-153(1980).
17. Spark, R.E. Review of gastrointestinal perfusion in treatment of uremia. Clin. Nephrol 11,81(1979).
18. Chang, T. M. S. Lancet II, 1371-1372 (1972).
19. Kjellstrand, C., Borges, H., Pru, C., Gardner, D., and Fink, D. Trans. Am. Soc. Artif. Intern. Org., 27, 24-30 (1981).

20. Drukker, W., Parsons, F.M., and Maher, J.F. Replacement of Renal Function by Dialysis. Martinus Nijhoff Publishers, Boston (1983).

21. Friedman, E.A., Saltsman, M.J., Beyer, M.M., and Josephson, A.S. Kid. Int., 10: S273-S276. (1976).

22. Scoot, B., Mulrooney, H., Stuart, P., and Haussinger, R. P. J. Gen. Microbiol. 135,1769-1776(1989).

23. Mobley, H. L. and Haussinger, R. P. Microbiol. Rev. 53, 85-108(1989).

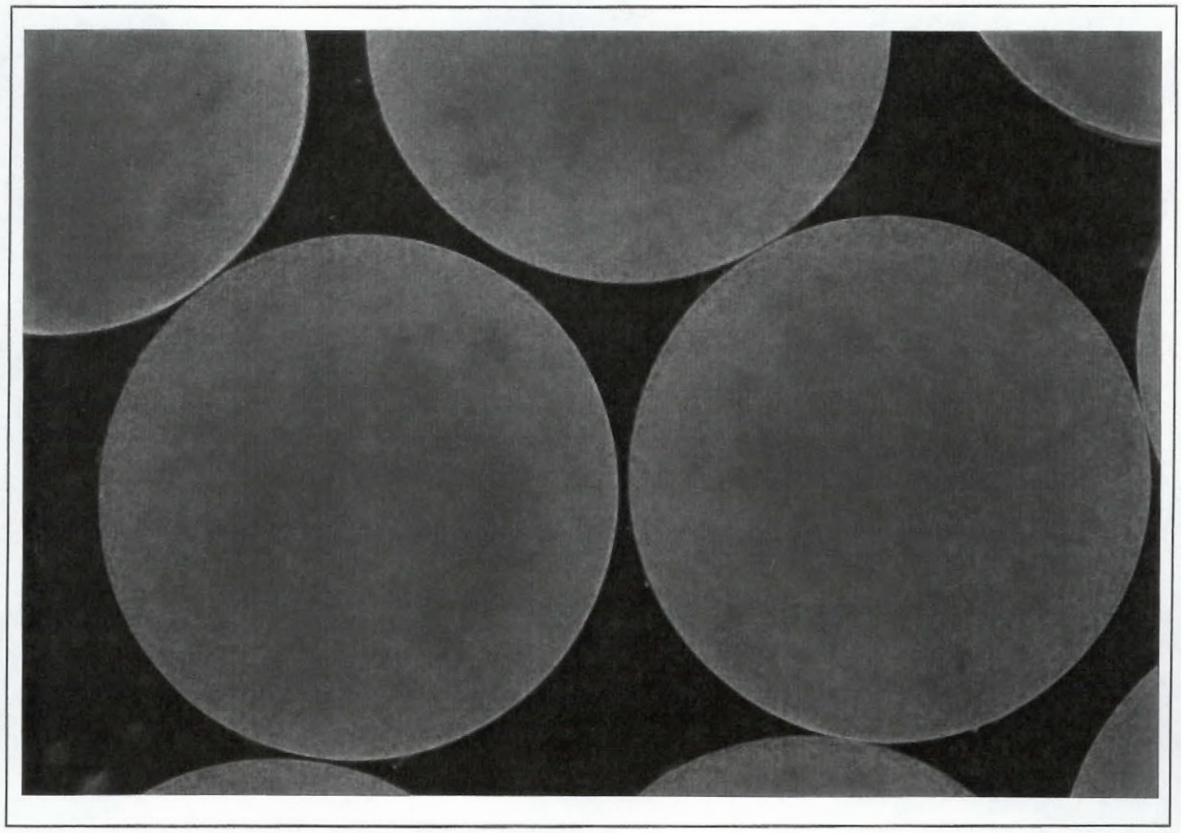


Figure 6.1: Photomicrograph of the alginate-poly-L-lysine-alginate (APA) microcapsules containing genetically engineered bacteria *E. coli* DH5 cells. Size 500 $\pm$ 45 μm in saline under the light microscope.

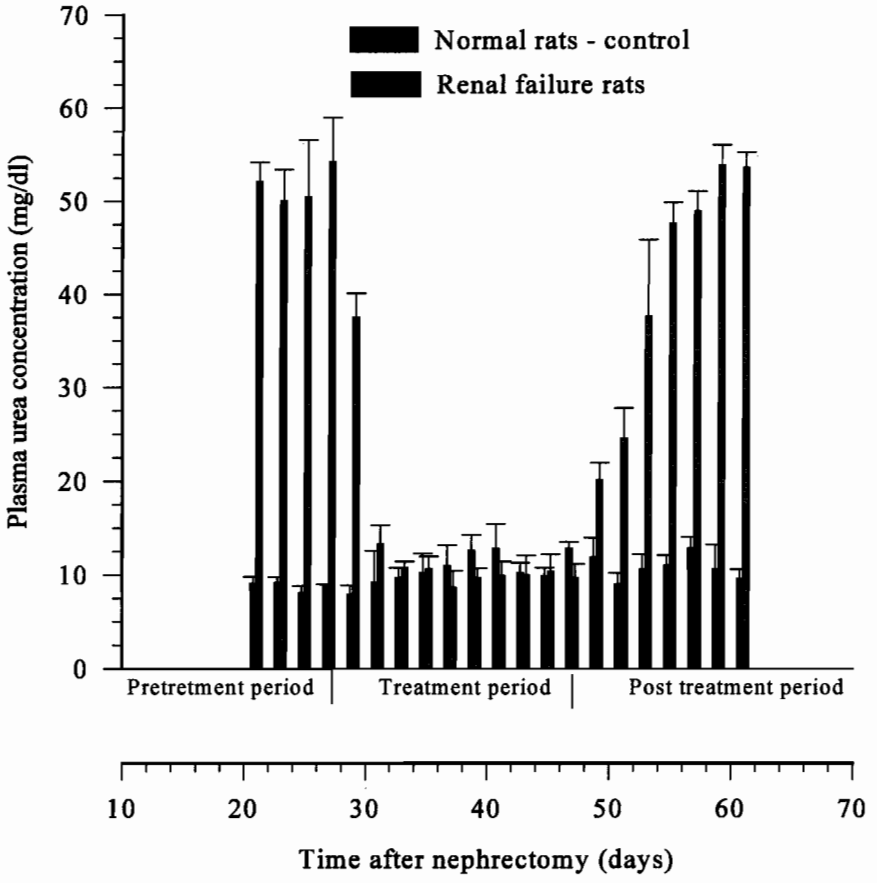


Figure 6. 2: Urea profile of uremic rat model before, during, and after treatment using microencapsulated genetically engineered bacteria.

6.5 NOTES

6.5.1 Notes for Figure 6.1, the photomicrograph of the alginate-poly-L-lysine-alginate (APA) microcapsules containing genetically engineered bacteria *E. coli* DH5 cells. Size 500 ± 45 μm in saline under light microscope.

Bacterial cells were obtained from Prof. R. P. Haussinger [22,23]. The details of the microencapsulation procedure is described elsewhere [12]. Briefly, log phase cells were suspended in an autoclaved 2 % sodium alginate in 0.9 % sodium chloride solution. The viscous alginate bacterial suspension was pressed through a 23-gauge needle using a syringe pump (Compact Infusion Pump). Compressed air through a 16-gauge needle was used to shear the droplets coming from the tip of the 23-gauge needle. The droplet was allowed to gel for 15 min in a gentle stirred ice-cold solution of 1.4 % calcium chloride. After gelation the bacterial alginate gel beads were coated with poly-L-lysine (0.05 % in HEPES, pH 7.20 for 10 minutes. The beads were then washed with HEPES pH 7.20 and coated with 0.01 % alginate solution for 4 minutes and called APA microcapsules. The microcapsules formed had an average diameter 500 ± 45 μm, stored at 4-8 ^{0}C and used in oral administration experiments.

6.5.2 Notes for Figure 6.2, the urea profile of uremic rat models and before, during and after the treatment using microencapsulated genetically engineered bacteria.

To prepare the uremic rat model, the anaesthetised animal is placed in ventral recumbence with its tail towards the surgeon. The hair in the right dorsal lumber area is clipped and the skin swabbed thoroughly with surgical scrub. A 2-3 cm incision is made into the skin caudal to the rib cage on the right side of the animal. A 2-3 cm incision is then made into the underlying muscle wall. The kidney is pulled through the muscle wall, the

renal artery, vein and ureter are ligated and the kidney is removed by incising the vessels and ureter between the kidney and the ligature remaining tissue is returned to the peritoneal cavity and the muscle wall is sutured. The skin incision is closed with 2-3 wound clips. The left side of the rat is prepared as if to perform a left nephrectomy. After an incision (2-3 cm) is made in the muscle wall, the left renal artery, vein, and ureter are located. Using a blunt forceps, the left renal vessels and ureter are isolated and separated from the peritoneal connective tissue. The renal vessels and ureter are ligated using a sterile silk suture. The muscle wall is sutured. The skin incision is closed with 2-3 metal wound clips.

Plasma urea was analyzed using quantitative urea determination kit (Sigma Chemical Co. USA). Plasma ammonia was analyzed using a fluorescent light scattering Multistat III microcentrifugal analyzer.

Statistical analysis was done by using Microsoft Excel statistical package, using student t-test.

CHAPTER 7

Artificial Cells Encapsulating Genetically Engineered Bacterial Cells for Biomedical Applications: Use of Microencapsulated Genetically Engineered *E. coli* DH5 Cells for the Removal of Systemic Urea and Ammonia in Uremic Rat.

S. Prakash and T.M.S. Chang

Manuscript in preparation.

7.1 ABSTRACT

This article describes in detail a new concept whereby microencapsulated genetically engineered microorganisms can be used for clinical applications by oral administration. The problem of urea and ammonia removal from the body fluid compartment in the diseased state is specifically addressed in this article. A daily oral administration of 5 mg of encapsulated bacteria to uremic rats results in a decrease of plasma urea from 52.08 $\pm$ 2.37 mg /dl to 37.53 $\pm$ 3.317 mg/dl within the first 48 hours, after this plasma level decreased to 10.58 $\pm$ 0.85 mg/dl from 52.08 $\pm$ 2.07 mg/dl on the day 7 and remains at this level throughout the treatment period. The oral administration of alginate-poly-L-lysine-algiante (APA) microencapsulated genetically engineered *E. coli* DH5 cell is a potentially efficient method for removing urea without producing ammonia in the experimental uremic rat model. To our knowledge, this approach has the ability to lower plasma urea and ammonia better than any other existing systems.

7.2 INTRODUCTION

Recent advances in molecular biology have furnished a variety of genetically engineered micorganisms with many specialised functions. However, their use to treat patients is limited. Our goal is to provide a simple system that will serve as an example whereby, one can test the use of genetically engineered microorganisms for clinical application. This study, therefore, is a model study. This study specifically addresses the use of microencapsulated genetically engineered bacteria *E. coli* DH5 cells for the removal of urea and ammonia. Urea and ammonia removal is necessary in kidney failure and liver failure, respectively. Also, elevated levels of urea and ammonia are seen in many other diseases. Uremia is caused by insufficient kidney function (Ashaba et al.,

1980; Castelman et al.,1974; Cohen, 1972; Drukker et al., 1983). As kidneys fail, substances normally excreted in the urine are retained in the blood and body tissues. This results in an increased concentration of waste metabolites (Ashaba et al., 1980; Castelman et al., 1974; Drukker et al., 1983; Friedman et al., 1974). For example, blood urea nitrogen (BUN) level increases from 15 mg % to 100-300 mg %, and serum creatinine increases from 1.0 mg % to 10-25 mg % (Charles et al., 1990). Similarly, elevated levels of ammonia in liver failure is evident (Cohen, 1972; Charles et al., 1990). These substances in high concentrations can result in severe disturbances of metabolic pathways (Ashaba et al., 1980; Cataneo and Chang, 1991; Cohen, 1972; Sparks et al., 1973; Guyton, 1995). Several attempts have been made to remove urea and ammonia. Very briefly, these methods can be divided into three categories : i) the use of adsorbents that binds urea directly (Ashaba et al., 1980; Esposito and Carmelo, 1980; Friedman et al., 1974; Walter et al., 1977), ii) the use of enzyme urease that breaks urea in to ammonia and its subsequent removal by chemical adsorbents (Chang, 1966; Charles et al., 1990; Dukker et al., 1983; Esposito et al., 1980; Freter et al., 1983; Friedman et al., 1974, 1978; Kjellstrand et al., 1981) and, iii) the use of microencapsulated multienzyme systems for the conversion of urea and ammonia into essential amino acids (Gu and Chang, 1988, 1991; Chang, 1991). In the first case, oxystarch, one of the adsorbents of urea, is extensively studied (Freter et al., 1983; Freidman et al., 1975; Esposito et al., 1980; Kolf et al., 1990; Sparks et al., 1972, 1975). However, the problem with this adsorbent is that a very large dose is required. The same problem of large dose requirement is encountered in urease-zirconium phosphate system (Castelman and McNeely, 1974; Chang, 1966, 1988, 1994; Esposito and Carmelo, 1980; Freter et al., 1983; Friedman et al., 1974; 1975). The third approach also lacks sufficient conversion rate (Gu and Chang, 1988, 1991). Thus, the need for efficient systems for urea and ammonia removal is clear.

The artificial cell concept, developed by Chang, provides a specialised environment for biologically active materials (Chang, 1964,1965,1980,1985,1990,1995; Lim and Sun, 80; Shian-Soon et al., 1994; Prakash and Chang, 1995; Sparks, 1979). We have shown that microencapsulated genetically engineered bacteria *E. coli* DH5 cells

have a very high efficiency to remove urea and ammonia from an artificial media (Prakash and Chang, ,1993,1995a,1995b,1996a; Chang and Prakash, 1995) and from the plasma in-vitro (Chang and Prakash, 1995; Prakash and Chang, ,1995a, 1995 b). In this paper, we report the details of our in-vivo studies on experimental uremic rat model that can be used for the removal of urea and ammonia during kidney failure, liver failure and other diseases.

7.3 MATERIALS AND METHODS

7.3.1 Chemicals

Alginic acid (low viscosity, Lot 611994) and poly-L-lysine (MW 16,100, Lot 11H5516) were purchased from Kelco and Sigma Chemical Co. (St. Louis, MO, USA) respectively. Unless otherwise specified, chemicals were obtained commercially of analytical reagent grade and not purified any further before use.

7.3.2 Microorganism and Culture Conditions

Genetically engineered bacteria *Escherichia coli* DH5, containing the urease gene from *Klebsiella aerogens*, was a generous gift from Prof. R. P. Haussinger (Mobley and Haussinger, 1989; Scoot et al., 1976). Luria-Bertani (LB) growth medium was used for primary cell cultivation (Sambrrok et al., 1989). The composition of LB medium was of 10.00 g/L bactotryptone (Difco), 5.00 g/L bacto yeast extract (Difco), and 10.00 g/L sodium chloride (Sigma). The pH was adjusted to 7.5 by adding about 1.00 ml of 1.00 N NaOH. Media were then sterilized in Castle Labclave for 30 minutes at 250^0 F. Incubation was carried out in 5.00 ml LB in 16.00 ml culture tubes at 37^0 C in an orbital shaker at 120 rpm. For large-scale production of biomass, for microencapsulation purpose, 250 ml Erlenmeyer flask containing 100 ml LB medium was used.

7.3.3 Microencapsulation Procedure

The details of the microencapsulation procedure is described elsewhere (Prakash and Chang, 1995 a; Chang and Prakash, 1995). Very briefly, microcapsules containing bacterium *E. coli* DH5 cells were prepared as follows: Bacterial cells are suspended in an autoclaved sodium alginate solution. The viscous alginate-bacterial suspension is pressed through a 23 gauge needle using a syringe pump (Compact Infusion Pump Model 975, Harvard App. Co. MA). Compressed air through a 16 gauge needle is used to shear the droplets coming from the tip of the 23 gauge needle. The droplets are allowed to gel for 15 minutes in a gently stirred ice-cold solution of calcium chloride (1.4 %). After gelation in the calcium chloride, alginate gel beads are coated with polylysine (0.05 % in HEPES buffer saline, pH 7.20) for 10 minutes. The beads are then washed with HEPES and coated with an alginate solution (0.1 %) for 4.00 minutes. The alginate-poly-L-lysine-alginate capsules were then washed in a 3.00 % citrate bath (3.00 % in 1:1 HEPES-buffer saline, pH 7.20) to liquefy the gel in the microcapsules. The microcapsules formed are stored at 4 ^{0} C and used for experiments.

7.3.4 Microcapsule Storage Conditions

After the microencapsulation, microcapsules were washed at least two times with sterile water. The microcapsules were resuspended in the Aggrobacteriium minimum broth (AG minimal media) at 4-10 ^{0} C (Marincs et al., 1994). This media, unlike L. B. media, does not support the growth of *E. coli*, it has however all the components which are necessary to maintain biochemical activity (Pat et al., 1993). Before use microcapsules were washed in normal saline and used for the experiments.

7.3.5 Reaction Media for In-Vitro Urea and Ammonia Removal Experiments

Reaction media in all experiments consisted of 1.00 g/L glucose, 20.00 mg/L

magnesium sulfate, 30.00 mg/L dipotassium monohydrogen phosphate, and 0.07 mg/L vitamin B_{12}. As required, filtered sterilized urea was added in the reaction media to make a urea concentration of 100 mg/dl. Plasma from a bovine source was used for plasma urea and ammonia removal experiments and filter-sterilized urea was added to the plasma. This was done to mimic the uremic patient's plasma characteristics in-vitro (Castelman et al., 1974).

7.3.6 Surgical Model of Uremia (Rat)

A surgical procedure for making uremic rat models was developed. For this male Wister rats of weight range 300-340 g were used. The details of the surgical procedure are described below:

7.3.6.1 Procedure

A two step procedure, one to perform right nephrectomy and the other to ligate the left artery, vein and ureter. The details of the involved two steps are as follows :

i) Unilateral (Right) Nephrectomy

The anesthetized animal is placed in ventral recombency with its tail towards the surgeon. The hair in the right dorsal lumber area is clipped and the skin swabbed thoroughly with a surgical scrub. A 2-3 cm incision is made into the skin caudal to the rib cage on the right side of the animal. A 2-3 cm incision is then made into the underlying muscle wall. The kidney is pulled through the incision of the muscle wall, the renal artery, vein and ureter are ligated and the kidney is removed by incising the vessels and ureter between the kidney and the ligature. The remaining tissue is returned to the peritoneal cavity and the muscle wall is sutured and skin incision closed.

ii) Unilateral (Left) Renal Artery / Vein / Ureter / Ligation

The left side of the rat is prepared as above. An incision (2-3 cm) is made in the muscle wall. The left renal artery, vein, and ureter are located. Using blunt forceps, the left renal vessels and ureter are isolated and separated from the peritoneal connective tissue. The renal vessels and ureter are ligated using sterile silk suture. The muscle wall is sutured. The skin incision is closed with 2-3 metal wound clips.

7.3.7 Experimental Procedure

The bacteria were grown in L B medium. Log phase bacterial cells were harvested by centrifuging at 10,000 g for 20 min. at 4 ^{0} C. The cell mass was then washed five times with sterile cold water to remove media components. Cells were then weighed and used for the urea and ammonia removal studies by free bacteria. For the microencapsulated urea and ammonia removal studies an equivalent mass of the cells were microencapsulated and used. In all in-vitro studies, reactions were performed in 250-ml Erlenmeyer flasks at 30 ^{0} C and 100 rpm, unless otherwise mentioned. The Lab-Line orbital environ-shaker equipped with thermal control and air quality were used for this purpose. Sampling was carried out aseptically at designated times. Bacterial cells, in the free bacteria removal studies, were removed from the sample by centrifugation at 15,000 rpm and the supernatant analyzed. For plasma urea and ammonia removal studies, we used bovine plasma with filter-sterilized urea added to it to a final urea level comparable with that of kidney failure patients (Castelman et al., 1974). In all the experiment, the ratio of the reactor volume to the amount of microencapsulated bacteria used was held constant.

For in-vivo animal studies, microcapsules containing log phase genetically engineered bacteria were first suspended in 0.8-1.0 ml sterile normal saline in a 5 ml syringe. The floating microcapsules were then force-fed orally using a curved 12G-3 1/2 stainless steel tube. Blood sampling was done by taking blood samples out from the rat

tail after sedating them using appropriate amounts of sedatives that have been reported not to have any side effects on renal or hepatic functions. The sedatives used were atravet (acepromazine) and ketaset (ketamine) in concentrations of 75 mg / kg and 5-10 mg / 9 kg intramuscularly, respectively. Blood was withdrawn using a small 23 G1 precision Glide needle and 10 ml syringe. Blood samples were then centrifuged immediately, using a eppendroff, and plasma was collected and analyzed for urea and ammonia concentrations.

7.3.8 Urea and Ammonia Determination

Urea concentrations were determined based on quantitative measurements in blood using the BUN kit purchased from Sigma Chemical Co. USA (Walker et al., 1977). Ammonia was analyzed using the light scattering Multistat III microcentrifugal analyzer. This was based on the reductive amination of 2-oxoglutarate, using glutarate dehydrogenase (GLDH), and reduced nicotinamide adenine dinucleotide (NADH). The decrease in absorbance at 340 nm due to the oxidation of NADH is proportional to ammonia concentration.

7.4 RESULTS

This study was designed to explore the use of microencapsulated genetically engineered microorganisms for the treatment of various diseases by oral feeding. This study more specifically assesses the future potential of encapsulated genetically engineered bacterial cells for the treatment of kidney failure, liver failure and other diseases in clinical practice.

Our earlier in-vitro studies demonstrated that alginate-polylysine-alginate (APA) encapsulated genetically engineered *E. coli* DH5 cells are very effective for removing

urea and ammonia both from reaction media and plasma (Chang and Prakash, 1995; Prakash and Chang, 1991, 1995 a, 1995 b, 1996a). The details of the involved process parameters in preparing microcapsules and in-vitro kinetics of urea and ammonia removals are described elsewhere (Prakash and Chang, 1995a, 1995b).

This paper describes the feasibility studies for this approach in an animal model. We designed a procedure to make a suitable uremic rat model. Results show (Figure 7.1) that this uremic rat model has a very high level of plasma urea compared to normal rats. This model is based on right kidney nephrectomy followed by left kidney unilateral left artery, vein, and ureter ligation.

Experiments were designed to evaluate the use of microencapsulated genetically engineered cells for the removal of urea. The experimental uremic rat model described earlier was used for this study. Besides monitoring pre-treatment and post-treatment urea levels in experimental rats as a self control, we use two other controls. One is normal rats. The other is uremic rats receiving control microcapsules containing no bacteria. In all experimental studies both normal and uremic rats were fed with regular rat chow. A quantity of 5 mg of log phase bacteria in alginate-poly-L-algiante microencapsules were fed using lavage tube daily to a group of 26 days old nephrectomized rats with an elevated level of plasma urea; a plasma urea concentration of 52.08 ± 2.06 % mg compared to normal rat plasma urea level which is 9.10 ± 0.71 % mg. Results in Figure 7.2 show that bacteria were able to lower plasma urea concentration from 52.08 ± 2.06 to 37.53 ± 3.31 mg/dl in the first 48 hours of treatment and to 13.34 mg/dl the day after. When we continue daily feeding treatment, we were able to maintain plasma urea level in uremic rats close to normal values for a period of 21 days. A return to high urea level is observed when the treatment was stopped. Urea level increased to 20.11 ± 1.8 % mg, on the next day followed by 24.52 ± 3.25 % mg, 37.60 ± 8.21 % mg, 47.57 ± 2.26 % mg, 48.92 ± 2.09 % mg, 53.80 ± 2.18 % mg, and 53.69 ± 2.59 % mg on days 2, 3, 4, 5, 6, and the day 7, respectively. Two animals died on day 8 of cessation of treatment. A dissection study showed a typical uremic death with symptoms of hemorragic gut,

conjugated and collapsed lungs, and the chest cavity filled with fluid. The urea concentration in the lung fluid raised to as high as 177.21 ± 12.90 mg/dl (n=2). Further, 50 % of all the animals died within the first 29 days of the cessation of treatment (Figure 7.2).

Analogous experiments were done to observe the fate of ammonia during urea removal by APA encapsulated bacteria. This was also done to evaluate the possible use of this system in liver failure. Results (Figure 7.3) show that during the control period the ammonia levels in the uremic rats were in the range of 539 ± 51 μM/L. Oral administration of microencapsulated bacteria decreased the level to 144 ± 24.70 μM/L and remained constant in the entire period of treatment. The ammonia level increased when the treatment was stopped.

The data obtained in Figure 7.4 show that free bacteria were also able to deplete plasma urea. During the experiment with free bacteria, the amount of bacteria used was the same as in the APA microencapsulated studies. When we compare the urea removal kinetics of free bacteria with APA encapsulated bacteria (Figure 7.5) we found that the overall kinetics to be similar. However, at the outset, the rate of urea removal by free bacteria was significantly smaller than that by APA encapsulated bacteria. Free bacteria lowered the systemic urea level by 20.28 ± 1. 06 % on the first day and 68.29 ± 4.30 % on the second compared to 36.34 ± 4.70 % and 80.49 ± 2.96 % by APA-encapsulated bacteria. Furthermore, during the post treatment period the rate of increase of plasma urea concentration in APA encapsulated bacteria is much higher than that observed with free bacteria. This shows that microencapsulated bacteria is excreted rapidly with the stool, whereas the part of free bacteria is retained in the intestine.

We calculated the urea removal by APA encapsulated genetically engineered bacteria and compared it with other available oral adsorbents in figure 7.6. We found that APA encapsulated bacteria were able to remove 3.20 ± 0.67 mg of urea / mg of biomass on day two and 8.00 ± 1.39 mg / mg of biomass on day four, whereas, oxystarch and

urease-zirconium-phosphate at their best can remove only 0.103 mg of urea / mg oxystarch and 0.033 mg of urea / mg of urease-zirconium-phosphate (Cohen et al., 1972; Gu and Chang, 1980; 1990; Esposito and Carmelo, 1980; Drukker et al., 1983; Friedman, 1975; Freidman et al., 1975; Kolff et al., 1990). This shows that microencapsulated genetically engineered bacterial system has several fold higher urea removal efficiency.

7.5 DISCUSSION

Among the current methods for the protection of cells, enzyme and biologicals, the use of artificial cell discovered by Chang at McGill university, is getting increasingly more attention (Chang, 1964, 1966, 1985, 1988, 1990, 1995; Gu and Chang, 1988, 1991; Lim and Sun, 1980; Shian-Soon et al., 1994). Its most recent example is in the immunosolation of islets by Sun (Lim and Sun, 1980) and clinical trial of microencapsulated islet conducted by Patric Soon-Shiang (Shian-Soon et al., 1994). The main objective of present study is to further explore the potentials of this novel process to treat various diseases using genetically engineered microorganisms. The specific objective of this research is to find a suitable means to manage urea and ammonia levels in uremia, kidney failure, liver failure and other diseases. Accordingly, we microencapsulated genetically engineered *E. coli* DH5 bacteria that contain urea utilizing the urease gene from *Klebsiella aerogens.*

The hypothesis of using bacteria to treat human diseases was suggested by Metchnikoff in 1908 (Sarvedra et al., 1995). However, very little attention has been given to this and only a few published reports are available. Setala et al. (1972,1973) reported the human clinical trial of urea utilizing bacteria for uremia patients by oral feeding. However, the bacteria they used did not have sufficient efficiency (Setala et al., 1972, 1973). Recently Sarvedra et al. (1995) of John Hopkins University School of Medicine used oral feeding of bacterium *Bifidobacterium bifidum* and *Streptococcus thermophilus*

to infants to prevent diarrhea (Sarvedra et al., 1995). However, this is mainly to change the bacterial flora only and not to have urea removal functions.

For the first time we report that the oral feeding of both microencapsulated and free bacteria reduces plasma urea in experimental uremic rat models. Our present study support the findings of our previous in-vitro studies about the efficiency of genetically engineered bacteria *E. coli* DH5 cells (Chang and Prakash, 1995; Prakash and Chang, 1991, 1995a, 1995b, 1996a). The in-vitro urea removal rate is detected higher compared to in-vivo plasma urea removal rate. When we compared, however, we found that encapsulated bacteria can, in-vivo, remove urea with much greater efficiency than the generally used oxystarch and urease-zirconium phosphate. Encapsulated bacteria remove 3.20 ± 0.67 mg of urea / mg of biomass on day second and 8.00 ± 1.39 mg / mg of biomass on day. Oxystarch and Urease-zirconium-phosphate, on the other hand, had the capacity to remove only 0.103 mg of urea / mg oxystarch and 0.033 mg of urea / mg of urease-zirconium-phosphate (Cataneo and Chang, 1991; Chang, 1972; Sparks et al., 1972,1979; Friedman, 1978; Drukker et al., 1983; Kjellstrand et al., 1981). Also the current oxystarch and urease-z.p. system works under specialized conditions which is not necessarily available during the actual treatment. For example, oxystarch has a good capacity for urea and ammonia binding only at a specific pH (Chang, 1972, 1985, 1995; Cohen, 1972; Friedman, 1978; Sparks, 1979; Kolff, 1990).

Quick removal of ammonia from the body fluid compartment is required in liver failure (Chang, 1976, 1980; Gu and Chang, 1988, 1991; Charles et al., 1990; Espostio et al., 1980; Friedman, 1995). In this study we have shown that ammonia removal is possible by encapsulated bacteria. Plasma ammonia concentration of uremic rats fell from 519 ± 51 μM/L to 144 ± 24.70 μM/L during urea removal experiments. This is a decrease of about 73 %. The overall kinetics of ammonia removal in this in-vivo study were similar to that of our earlier in-vitro studies (Chang and Prakash, 1995; Prakash and Chang, 1995a, 1995b, 1996a).

The behavior of the microencapsulated bacteria and free bacteria in terms of urea removal efficiency (Figure 7.5) indicates clear advantages of using encapsulated bacteria over free bacteria. At the onset of the treatment period, free bacteria have lower urea depletion capacity than micoencapsulated bacteria. This is because of the death of some unprotected free bacteria during the gastrointestinal passage. This result is in agreement with the earlier report of Freter et al. in 1983, where they conclude that one only have a maximum of 90 % *E. coli* using a bicarbonate pH neutralizing buffer to protect for cell death (Freter et al., 1983). A 10 % death is evident in feeding of free *E. coli* (Freter et al., 1983). Furthermore, figure 7.5 shows that when treatment is stopped free bacteria last longer in terms of urea lowering efficiency, than encapsulated bacteria. This means that the free bacteria are retained in the intestine and may disturb the natural flora of the intestine. In the case of microencapsulated bacteria, they were excreted with the stool.

This study shows the therapeutic feasibility of oral feeding of microencapsulated genetically engineered bacteria *E. coli* DH5 for the removal of urea and ammonia. This study also provides the foundation for the development of oral microcapsules containing other type of genetically engineered microorganisms for the removal other unwanted metabolites.

7.6 CONCLUSIONS

This study describes a new method for removal of urea and ammonia by oral feeding of microencapsulated genetically engineered bacterial cells. The microencapsulated genetically engineered *E. coli* DH5 cells efficiently lower urea from the body fluid compartments of the uremic rat model. Urea depletion was unaffected by the microencapsulation process. Both free and encapsulated bacteria are effective in urea removal. Encapsulated bacteria were able to reduce 36.34 ± 4.70 % of the total plasma urea within the first 48 hours and 80.49 ± 2.96 % of plasma urea on the second day.

Under similar conditions, microencapsulated bacteria can lower plasma ammonia from 539 μM to 144.00 ± 24.70 μM in the 48 hours of the treatment. Bacterial cells do not produce ammonia and use urea for their metabolic nitrogen requirement. Our study shows that this novel approach is several hundred fold more efficient in removing urea and ammonia than the standard oxystarch and urease-zirconium phosphate approaches.

7.7 ACKNOWLEDGEMENTS

T MSC gratefully acknowledge the research support of the Medical Research Council of Canada and the "Virage" award of the Quebec Ministry of Higher Education Science and Technology "Center for Excellence in Biotechnology". SP acknowledges the National Overseas Scholarship from the Government of India for completing his Ph.D. in Biomedical Engineering at McGill University. The generous gift of *E. coli* DH 5 used in this study by Prof. R P. Haussinger is gratefully acknowledged.

7.8 REFERENCES

Ashaba, H., Bergstrom, J., Furst, Peter, F, Christina, J., and Yahiel, V. (1980). Plasma middle molecules in asymptomatic and sick uremic patients. In : Middle molecules in uremia and other diseases. (Hort Klinkmann et al. ed.),137-141.

Castelman, B. and McNeely, B. U. (1974). Case records of the Massachusetts General Hospital. N. Engl. J. of Med., 290, 39-49.

Cattaneo, M., Chang, T. M. S. (1991). The potential of microencapsulated urease-zeolite oral sorbent for the removal of urea in uremia. J. Am. Soc. Artif. Intern. Org., 37, 80-87.

Chang, T.M.S. (1964). Semipermeable microcapsules. Science. 146, 524-525.

Chang, T.M.S. (1966). Semipermeable aqueous microcapsule (artificial cells) With emphasis on experiments in extracorporeal shunt systems Trans. Am. Soc. Artifi. Intern. Org., 12, 13-19.

Chang, T.M.S. (1980). New approaches using immobilized enzymes for the removal of urea and ammonia. Enzyme Eng., 5, 225-229.

Chang, T.M.S. (1985).Biotechnology of artificial cells including application to artificial organs, In: M. Moo Young (ed.), Comprehensive biotechnology: The principle applications and regulation of biotechnology in industry, agriculture, and medicine. Pergamon press, New York, 53-73.

Chang, T.M.S. (1988). Methods in the medical application of immobilized proteins, enzymes and cells. Methods in Enzymology, 137, 444-457.

Chang, T.M.S. (1990). Biotechnological and medical application of immobilization of hepatocytes, microorganisms and enzyme systems by microencapsulation in artificial cells. Enz. Eng., 10, 331-339.

Chang, T.M.S. (1993). Living cells and microorganisms immobilized by microencapsulation inside artificial cells, In: M. F. Goosen (ed.), Fundamentals of animal cell encapsulation and immobilization. CRC press, Boca Raton, FL. 183-94.

Chang, T.M.S. and Prakash, S. (1995). Artificial cells and genetically engineered microencapsulated for *E. coli* cells, for urea and ammonia removal: "Methods in Molecular Biology: volume on Expression and Detection of Recombinant Gene" Series, Humana Press, USA Chapter 75 (in press).

Cohen, B.D. (1972). Uremic toxins. In : Uremia, an international conference on pathogenesis, diagnosis and therapy, 1-11.

Charles, S., David, M., Stacia, M. (1990). In : Deborah, A. (ed.), Human physiology: foundations and frontiers. Times mirror college publi., St. Louis, 526-527

Drukker, W., Parsons, F. M., and Mahar, J. F. (1983). Replacement of renal functions by Dialysis. Martinus Nijhoff, Boston.

Esposito, R., Carmelo, G. (1980). Polyhalides. In: G. Caramelo (ed.), Sorbents and their clinical applications. Academic press, London,133-153.

Freter, R., Brickner, H., Fekete, J., Vicraman, M., M., and Carey, E. (1983). Survival and implantation of *E. coli* in the intestinal tract. Infection and Immunity, 39, 686-703.

Friedman, E.A., Fastcook, J., Beyer, M. M., Rattazzi, T., and Josephson, A. S (1974). Trans. Am. Soc. Artf. Int Organs, 20, 161.

Friedman, E.A., Laungani, G. B., Beyer, M. M. (1975). Kidney Int., 7, 337-379 .

Friedman, E.A. (1978). In: Friedman, E. A. (ed.), Strategy in renal failure. Willey, New York. 521-528.

Gu, K.F., and Chang, T.M.S. (1988). Conversion of alpha-ketoglutarate into L-glutamic acid with urea as ammonia source using multienzyme system and NAD immobilized by microencapsulation with artificial cell in bioreactor. Biotechnol. and Bioeng., 32, 363-368.

Gu, K.F. and Chang, T.M.S. (1991). Conversion of urea or ammonia in to essential amino acids, L-leucine, L-valine and L-isoleucine using artificial cells containing an immobilized multienzyme system and dextran-NAD. Yeast alcohol dehydrogenase for enzyme recycling. J. Biotech. Applied Biochem., 12,27-236.

Guyton, A. C. (1995). In : Textbook of medical physiology. 9th ed. Philadelphia, W.B. Saunders, Publications, USA.

Kjellstrand, G., Borger, H. Pru, C.G. and Fink, D. (1981). Trans. Am. Soc. of Artif. Intern. Org., 27, 24-30.

Kolff, W. J. (1990). Artificial kidney and artificial heart: Future considerations. Int. J. Artif. Org., 13, 404-406.

Kwok, W.Y., Kiparissides, C., Yuet, P., Harris, T. J., and Goosen, M. F. (1991). Mathematical modeling of protein diffusion in microcapsules: A comparison with experimental results. Can. J. Chem. Eng., 69, 360-370.

Lim, F., and Sun, A.M. (1980). Microencapsulated islets as bioartificial pancreas. Science, 210, 908-910.

Marincs, F. and White, D.W.R. (1994). Immobilization of *E. coli* expressing the lux gene of Xenorhabdus luminescence; Applied and Environmental Microbiology. Oct., 3862-3863.

Mobley, H.L. and Haussinger, R. P. (1989). Microbial ureases: Significance, regulation, and molecular characterization. Microbiol. Rev., 53, 85-108.

Pat, M.L., Kong, H. L., and Yew, S. S. (1993). Covalent immobilization of aminoacylase to alginate for L-phenylalanine production. Chem. Tech. Biotechnol., 58, 65-70.

Prakash, S. and Chang, T. M .S. (1993). Microencapsulated genetically engineered *E. coli* cells containing genes form *K. aerogens* for urea and ammonia removal. Biomat. Artfi. Cells Immob. Biotechnol., 19, 687-697.

Prakash, S. and Chang, T. M. S. (1995a). Preparation and in-vitro analysis of genetically engineered *E. coli* DH5 cells, microencapsulated in artificial cells for urea and ammonia removal. Biotechnology and Bioengineering, Vol.46, 621-626.

Prakash, S and Chang, T. M .S. (1995b). Microencapsulated genetically engineered *E. coli* DH5 cells for urea and ammonia removal in kidney failure and liver failure: IEEE Transaction in Biomedical Engineering, (in press).

Prakash, S. and Chang, T.M.S. (1996a). A new approach of using artificial cells containing genetically engineered *E. coli* DH5 cells for urea and ammonia removals. Submitted to ASAIO journal.

Sarvedra, M. J., Baumann N., Oung, I., Perman, J. and Yolken R.H. (1995). Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhea and shedding of rotavirus. Lancet, 344, 1046-1049.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scoot, B., Mulrooney, H. Stuart, P., and Haussinger, R. P. (1989). Regulation of gene expression and cellular localization of cloned *K. aerogens* urease. J. Gen. Microbiol., 135, 1769-1776.

Setala, K., Heinonen, H., and Schreck-Purla (1973). Ingestion of lyophilized soil bacteria for alleviation of uremic symptoms. J. Int. Res. Comm. Syst., 73.

Setala, K., Heinonen, H., and Schreck-Purla (1972). Uremic waste recovery. Proc. Conf. EDTA, 9, 514-520.

Shian-Soon, P., Heintz, R., Yao, Z., Zheng, T., Murphy, M., Molony, M. K., Schmehl, M., Harris, M., Mendez, Robert, Mendez, Raephel, Standford, P. (1994). Insulin

independence in a type 1 diabetic patient after encapsulated islet transplant. Lancet, 343, 950-951.

Sparks, R.E., Saleman, R.M., Meier, P.M., Litt, M.H., and Lindon, O. (1972). Binders to remove metabolite from the GI tract. Trans. Am. Soc. Artif. Int. Org., 18, 458-464.

Sparks, R.E. (1979). Review of gastrointestinal perfusion in treatment of uremia. Clin. Nephrol., 11,81-86.

Symmour, A.C., Philip, P.C. (1954). Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem., 209,145-147.

Walker, J. M., Jacobson, R. L., Stephen, W. J., and Rose, D. (1977). The role of adsorbents in the wearable artificial kidney. In: Gilchrist, T. (ed.), Artificial Org. Macmillan, London, 137-149.

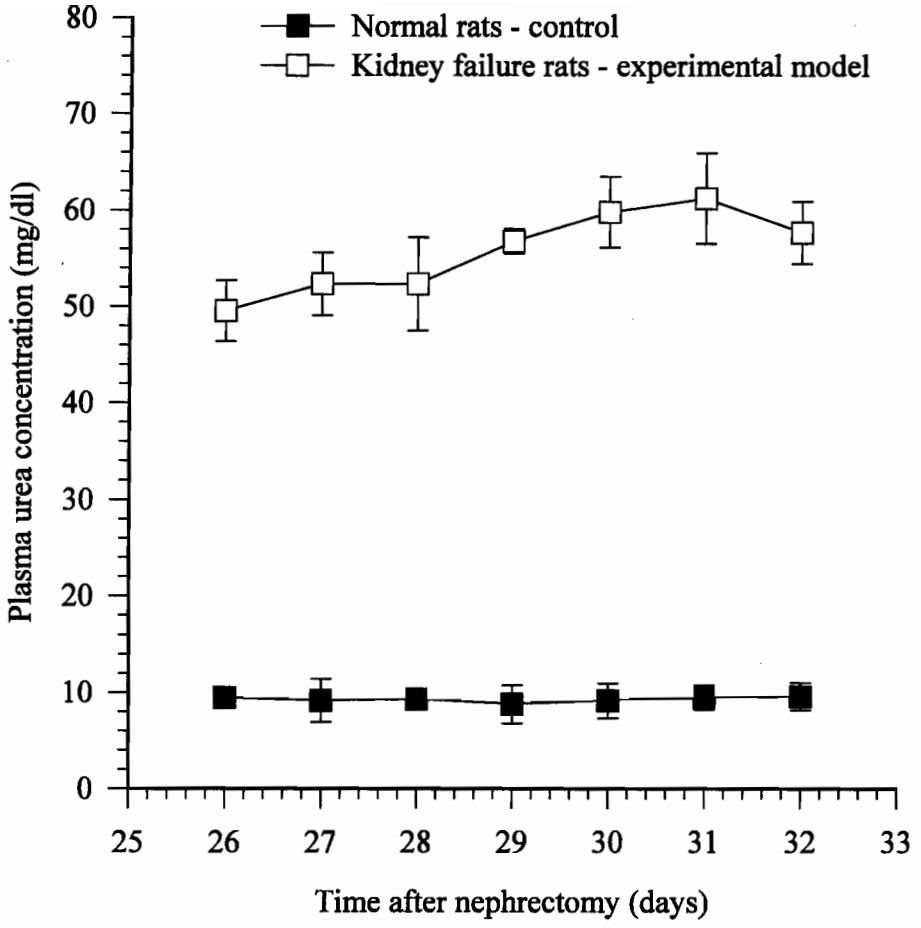


Figure 7.1: Plasma urea profile of uremic rat model : right nephrectomy and left renal artery / vein / ureter ligation.

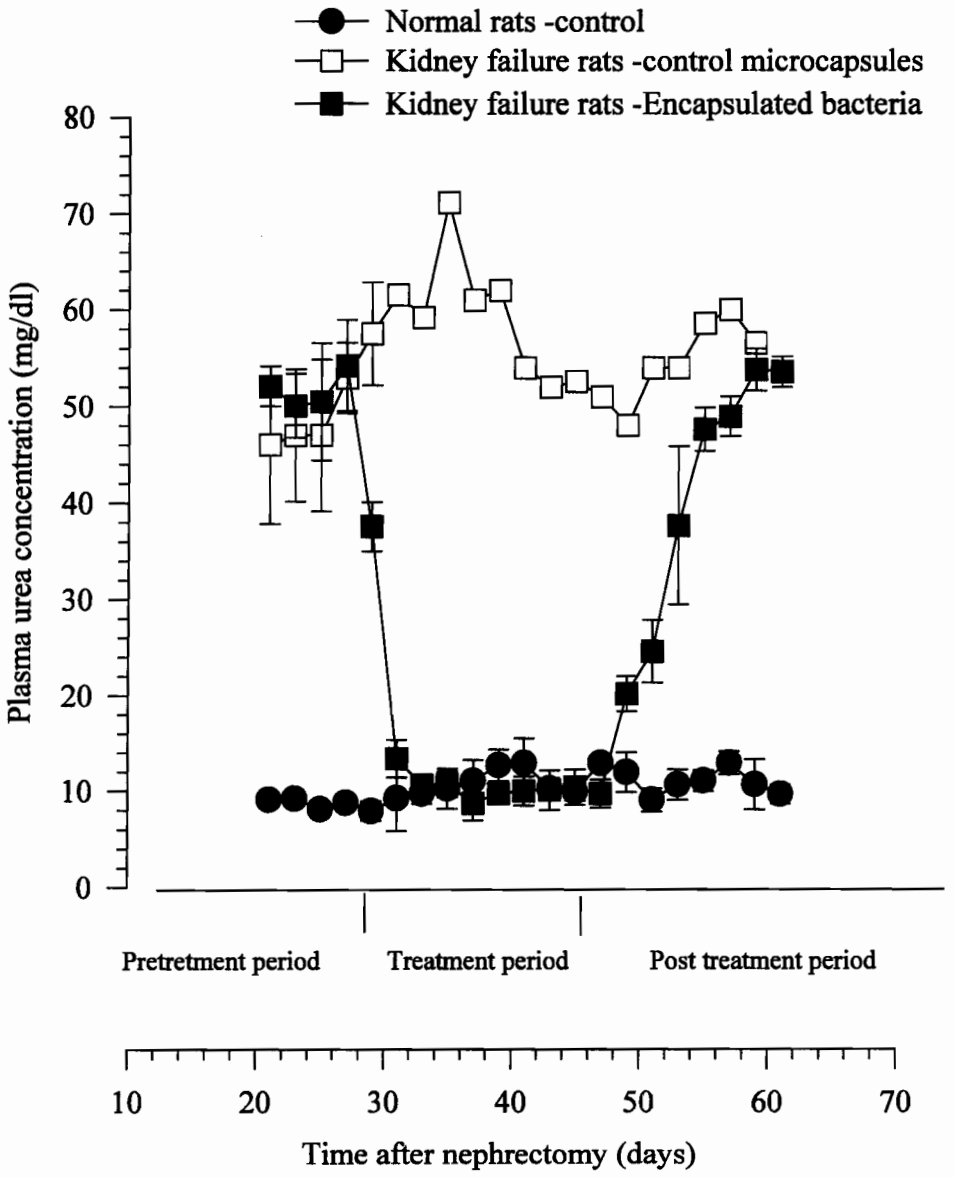


Figure 7.2: Plasma urea profile of uremic rats before, during, and after oral administration of APA-Encapsulated *E. coli* DH 5 cells. For kidney failure control rats no standard deviation after day 35 is reported because 50 % of the rats died.

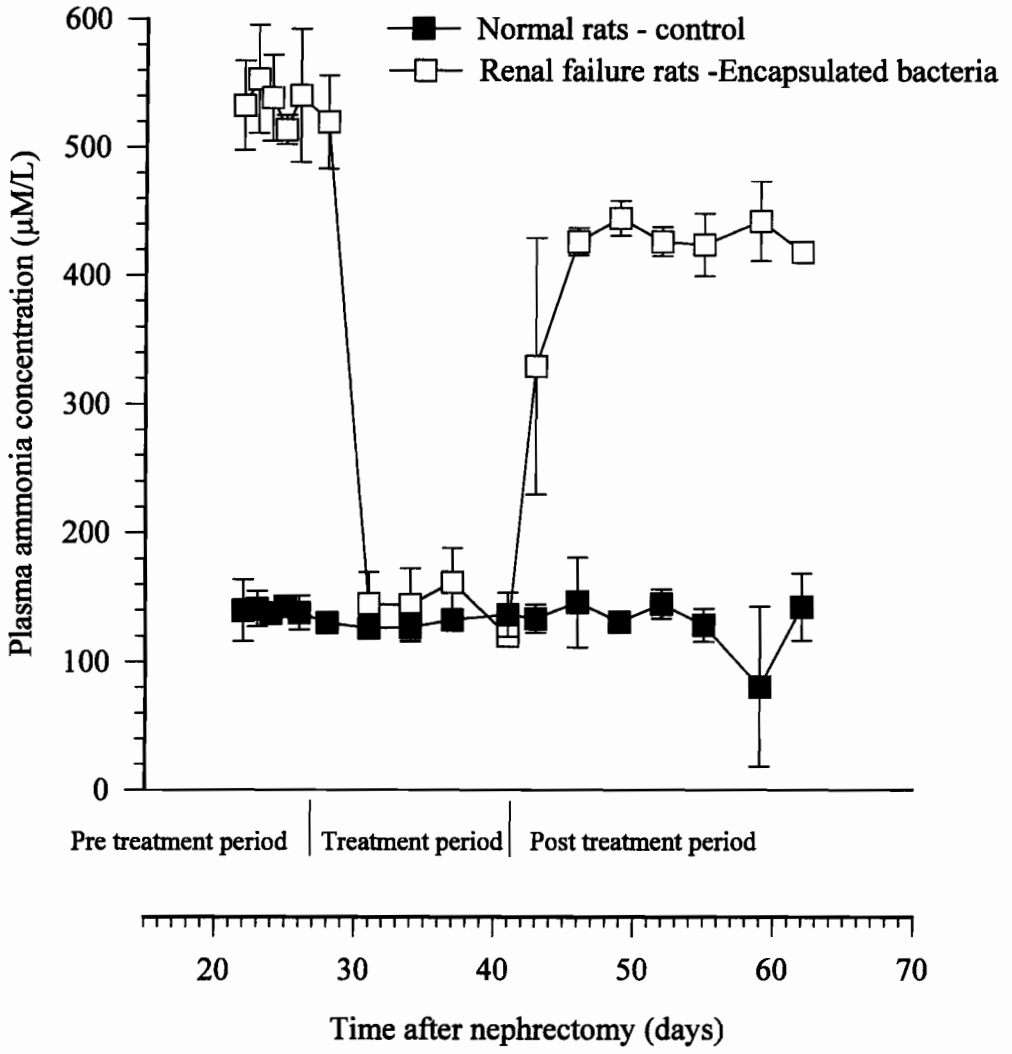


Figure 7.3: Plasma ammonia profile of uremic rats before, during and after treatment by oral feeding of APA-Encapsulated genetically engineered bacteria.

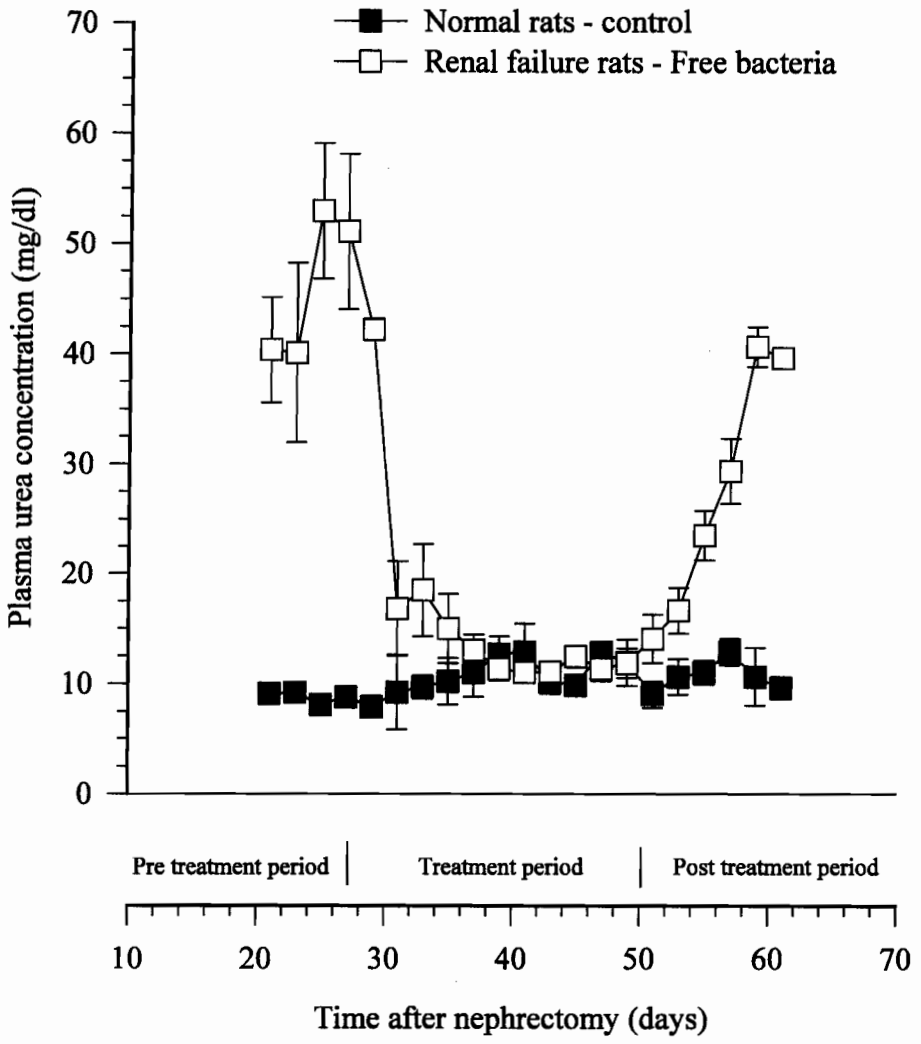


Figure 7.4: Plasma urea removal profile of experimental uremic rats before, during ,and after oral administration of free bacteria.

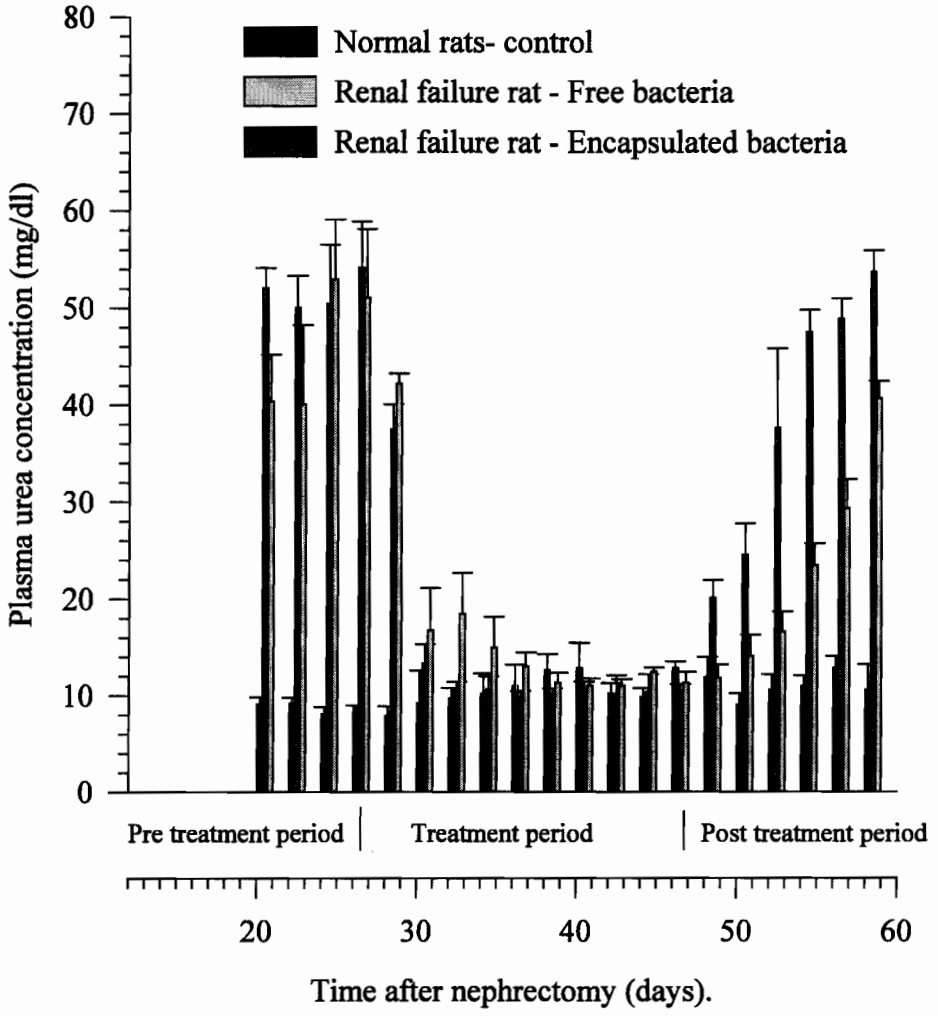


Figure 7.5: Comparative analysis of urea removal profile of free and APA-Encapsulated genetically engineered bacteria in experimental uremic rats.

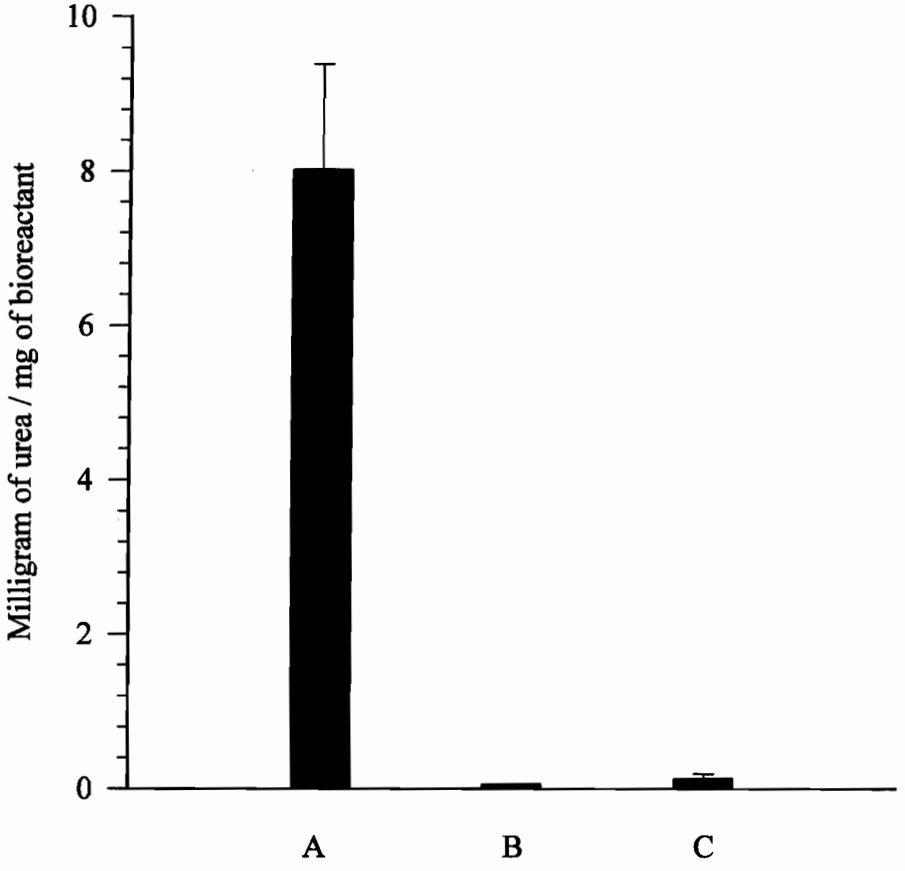


Figure 7.6: Comparative study.

A = Urea removal efficiency of APA-Encapsulated genetically engineered bacteria.

B = Urea removal capacity of urease-zirconium-phosphate system.

C = Urea removal capacity of oxystarch.

CHAPTER 8

SUMMARY
CONCLUSIONS
AND
CLAIMS TO ORIGINAL CONTRIBUTIONS

8.1 SUMMARY OF OBSERVATIONS

The marked * claims to the original contributions to knowledge.

(1)* Artificial cells containing genetically engineered bacteria *E. coli* DH 5 cells were found effective in removing urea and ammonia from the reaction media (Fig. 2.1 and 2.2). The APA-encapsulated bacteria were able to reduce 100.00 ± 1.00 mg / dl of urea to 1.50 ± 0.0013 mg / dl in 30 minutes when 100 mg of cells were used. The same APA encapsulated bacteria, under similar conditions, were able to lower ammonia from 758.00 ± 70.00 μM to 90.42 ± 38.05 μM in 20 minutes. The bacteria do not produce ammonia during urea utilization.

(2)* Urea and ammonia removal kinetics of both free and APA-encapsulated bacteria from reaction media were found to be similar (Fig.2.3). The urea depletion efficiency was, therefore, not affected by the microencapsulation process.

(3)* The complete microencapsulation process parameters were optimised for genetically engineered bacterial cell immobilization. The alginate concentration (Table 2.1), air flow rate (Fig. 3.2), and liquid flow rate (Fig.3.3) were found to play a major role in the microencapsulation process by the drop technique (Fig. 3.1). A two percent (w/v) alginate concentration, a 0.0724 ml/min alginate-bacterial cell suspension flow rate, and 2.00 L/min. air flow rate produces spherical and sturdy APA microcapsules of 500 ± 45 μm in diameter. These microcapsules were found to be mechanically stable up to 210-rpm agitation speed; that is, no cell leakage occurs (Fig. 3.4). The alginate concentration, in the tested range did not affect the bacterial cell viability.

(4)* A new method for plasma urea removal by artificial cells containing genetically engineered bacterial cells is proposed and demonstrated (Fig. 3.5). Microencapsulated bacteria were able to reduce 87.89 ± 2.25 % of the total plasma urea level within 20 minutes and 99.99 % of urea in 30 minutes.

(5)* A new procedure for plasma ammonia removal by microencapsulated genetically engineered bacteria *E. coli* DH5 cells is proposed and demonstrated (Fig 3.6). APA encapsulated bacteria can lower the plasma ammonia level from 975.14 ± 70.15 μM to 81.15 ± 7.37 μM in 30 minutes.

(6)* APA microcapsules were found reusable for three cycles of plasma urea or ammonia removal before bacterial cell leakage might occur (Fig.3.9, 3.12, 3.13, 3.14, 3.15).

(7)* The in-vitro plasma urea removal results indicate that this new approach of plasma urea removal is 10 to 30 times more efficient for removing urea and ammonia than the standard approaches (Fig. 3.11). The amount of microencapsulated bacterial cell required, for a 70 kg man, is only 40.00 ± 8.60 g in order to remove 87.89 ± 2.25 % of the initial 100 ± 4.00 mg/dl plasma urea within 20 minutes and 99.99 % of the urea in 30 minutes (Fig 3.11). However, to achieve the same effect a quantity of 388.34 g oxystarch and 1211 g of urease-zirconium-phosphate are required.

(8)* A new continuous process using a column bioreactor for plasma urea and ammonia removal by APA membrane encapsulated genetically engineered bacteria *E. coli* DH5 cells is designed (Fig. 4.1). The feasibility of this column is also demonstrated (Fig. 4.2,4.4).

(9)* It is found that using this column bioreactor one can lower the urea concentration about 83.57 % in 20 min, 92.12 % in 30 min and 98.02 % in 60 min, under the conditions mentioned. The same column bioreactor is also very efficient in lowering plasma ammonia from 741 $\pm$ 25.00 to 26.96 $\pm$ 5.78 μM in 30 minutes, which is a 96 % reduction in plasma ammonia level.

(10)* The influence of initial plasma urea concentration on the proposed column bioreactor was evaluated to explore its application for various degrees of uremic patients. Urea concentrations of 45.84, 100.14, 115.26 and 186. 34 $mg.dl^{-1}$ were chosen for this study. A slower rate at higher plasma urea concentration was observed (Fig. 4.3). Similar experiments were designed for ammonia removal selecting plasma ammonia concentrations of 462 $\pm$ 8.26 μM, 741 $\pm$ 26.12 μM, and 985 $\pm$ 34.16 μM. Results show that the rate of ammonia removal is greater when the plasma ammonia concentration is lower (Fig. 4.5).

(11)* The operational stability of the column bioreactor was found secure only for three cycles (Fig. 4.5).

(12) A detailed study for column bioreactor plasma urea and ammonia removal approach is required.

(13)* A new surgical method for making a uremic rat model was designed. This was done surgically by performing right nephrectomy and left renal artery / vein / ureter / ligation. Results show (Fig. 7.1) that this uremic model has a very high plasma urea and ammonia levels and was found suitable for the intended purpose.

(14)* The in-vivo urea removal experiment, on the experimental rat model, shows (Fig. 6.2) that APA-Encapsulated bacteria are very effective in lowering plasma urea.

(15)* For in-vivo studies, a dosage of 11.15 ± 2.25 mg microencapsulated genetically engineered bacteria *E. coli* DH5 cells / kg of body weight was administered to uremic rats. The plasma urea concentration during the control period was as high as 52.08 ± 2.06 % mg . During the test period or treatment period, 21 days, the plasma urea levels were maintained in the normal range of 10.02 ± 2.5 % mg.
Upon discontinuation of oral administration, the plasma urea returned to a uremic level. The urea level went back to as high as 53.69 ± 2.59 % mg on day 7 (Fig.6.2). Two animals died on day 8 upon cessation of treatment. A dissection study showed a typical uremic death with symptoms of hemorrhagic gut, conjugated and collapsed lungs, and the chest cavity filled with fluid. Urea concentration in the lung fluid reached as high as 177.21 ± 12.90 mg/dl (n =2).

(16)* Results (Fig. 7.3) show that the plasma ammonia level after oral administration of encapsulated bacteria *E. coli* DH5 cells to uremic rats which was always present in the range of 545 ± 51 μM/L decreases to 144 ± 24.70 μM/L and remained constant during the entire period of treatment.

(17.)* The possible use of genetically engineered free bacteria for various applications was also explored. Figure 7.4 shows that free bacteria was also able to lower plasma urea. When the urea removal kinetics of free bacteria were compared with APA encapsulated bacteria (Fig. 7.5), we found that the overall kinetics was similar. However, at the outset, the rate of urea removal by free bacteria was much smaller than that of APA encapsulated bacteria. Free bacteria removes 20.28 ± 1. 06 % on the first day and 68.29 ± 4.30 % on the second compared to 36.34 ± 4.70 % and 80.49 ± 2.96 % by APA-encapsulated bacteria, respectively. Furthermore, during the post treatment period the rate of increase of plasma urea concentration in APA encapsulated bacteria is much higher than that

observed with free bacteria. When treatment is stopped, free bacteria last longer in terms of urea lowering efficiency, than encapsulated bacteria. The encapsulated system holds the advantage of being removed more quickly than the free system. The encapsulated bacteria, therefore, is recommended.

(18)* The urea removal efficiency of artificial cells containing genetically engineered bacteria *E. coli* DH5 cells in uremic rats were calculated, using a single pool model, and compared with other available counterpart oral adsorbents (Fig. 7.6). It is found that APA-encapsulated bacteria were able to remove 3.20 ± 0.67 mg of urea / mg of biomass on day two and 8.00 ± 1.39 mg / mg of biomass on day four, whereas, oxystarch and urease-zirconium-phosphate at their best can remove only 0.103 mg of urea / mg of oxystarch and 0.033 mg of urea / mg of urease-zirconium-phosphate. This shows a several fold superiority of the microencapsulated genetically engineered bacterial system over presently used urea removal systems.

8.2 CONCLUSIONS

A hypothesis whereby a new approach of using genetically engineered cells was made. This project was undertaken, therefore, to find a new and suitable way to use genetically engineered cells for various applications using the concept of artificial cells. Artificial cells containing genetically engineered *E. coli* DH5 cells for the removal of urea and ammonia were taken as an example to test this new hypothesis and its feasibility. This was also chosen as presently we do not have an effective urea and ammonia removal system in kidney failure, liver failure and other diseases.

After reviewing the results obtained the following conclusions can be made:

1. It is now possible to use genetically engineered bacterial cells more effectively for various applications, including therapeutics, biotechnology, and biomedical engineering applications, by using the concept of artificial cells.

2. A new approach for the removal of urea and ammonia by using artificial cells containing genetically engineered bacteria *E. coli* DH 5 cells has been proposed and its feasibility has been demonstrated in both in-vitro studies and in-vivo studies on experimental uremic rats. However, further study is required for the purpose.

3. This study confirms the lab scale feasibility of this new approach. The prospect of extending this new approach for many other types of genetically engineered cells is strong.