Form Approved Through 05/2004 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed character length restrictions indicated.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 56 characters, including spaces and punctuation.)*
Models and Simulations of Calcium Binding Sites

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — **New Investigator** ☒ **No** ☐ **Yes**

3a. NAME *(Last, first, middle)*
Eisenberg, Robert S.

3b. DEGREE(S)
Ph.D.

3c. POSITION TITLE
Professor and Chairman

3d. MAILING ADDRESS *(Street, city, state, zip code)*
1653 West Congress Parkway
Chicago, Illinois 60612

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Molecular Biophysics and Physiology

3f. MAJOR SUBDIVISION
NA

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (312) 942-6467 FAX: (312) 942-8711

E-MAIL ADDRESS:
beisenbe@rush.edu

4. HUMAN SUBJECTS RESEARCH
☒ No
☐ Yes

4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.
0000482

4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no.
A3120-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/05	06/30/10				

9. APPLICANT ORGANIZATION
Name Rush University Medical Center
Address 1653 West Congress Parkway

Institutional Profile File Number (if known) 6644301

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
1362174823A1
DUNS NO. 06-861-0245
Congressional District 07

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Jane Winger
Title Director, Fund Accounting
Address 1700 West Van Buren Street
Chicago, Illinois 60612
Tel: (312) 942-5622 FAX: (312) 942-4022
E-Mail: Jane_Winger@rush.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Donna J. Knuth, MBA
Title Director, Sponsored Projects
Address 1653 West Congress Parkway
Chicago, Illinois 60612
Tel: (312) 942-3354 FAX: (312) 942-6876
E-Mail: dknuth@rush.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.	SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*	DATE
15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*	DATE

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Channels are proteins with holes down their middle that control an enormous range of biological function in health and disease by controlling movement of charged atoms (ions) across otherwise insulating membranes. Ions are charged spheres that move through channels by diffusion and drift in the electric field. Open channels allow membranes to select between different kinds of ions: selectivity is a 'defining feature' of life, at least in textbooks. Channel structure does not change once they are open and so we can try to understand and control selectivity of channels using the language and mathematics of physical science, without addressing special properties of proteins or their conformation changes.

Channels have large amounts of permanent electrical charge on their walls, created by the natural charge on the amino acids forming the protein. The permanent charge must be accompanied by (nearly) equal amounts of opposite mobile charge. Ions and channels are inseparable, according to a basic law of electricity, called 'the principle of electroneutrality'. The number density (i.e., concentration) of ions in channels is very high, often ~20 M (pure water is ~55 M), so it is logical to think of ions in channels the way physical chemists think of ions in concentrated solutions. Surprisingly, such simple theories acccount for many complex highly selective properties of calcium channels without invoking other special forces that might be present. Evolution seems to use crowded charge to produce selectivity, more than anything else.

We propose to design highly selective calcium channels using models of crowded charge. We propose to build such channels using techniques of molecular genetics. We propose to measure ions moving through single channel molecules with the techniques of channel biophysics.

Preliminary work shows the feasibility of the proprosal. Constructed channels have many, but not all the properties of highly selective Ca channels from the heart. Theory suggests what needs to be improved.

PERFORMANCE SITE(S) *(organization, city, state)*

1) Rush University Medical Center, Chicago, Illinois
2) Biomade Technology Foundation, Groningen, The Netherlands

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Eisenberg, Robert	Rush University Medical Center	Principal Investigator
Gillespie, Dirk	Rush University Medical Center	Co- Principal Investigator
Meijberg, Wim	Biomade Technology	Co-Principal Investigator
Miedema, Henk	BiomadeTechnology	Senior Researcher
Tang, John	Rush University Medical Center	Assistant Professor
Vroienraets, Maarten	Biomade Technology	Research Scientist

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

Principal Investigator/Program Director (Last, First, Middle): Eisenberg, Robert S.

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT

TABLE OF CONTENTS

	Page Numbers
Face Page	1
Description, Performance Sites, and Personnel	2
Table of Contents	3
Detailed Budget for Initial Budget Period (or Modular Budget)	4
Budget for Entire Proposed Period of Support (not applicable with Modular Budget)	5
Budgets Pertaining to Consortium/Contractual Arrangements (not applicable with Modular Budget)	7-8
Biographical Sketch – Principal Investigator/Program Director (*Not to exceed four pages*)	9-11
Other Biographical Sketches (Not to exceed four pages for each – *See instructions*)	12-21
Resources	22-23
Research Plan	24
Introduction to Revised Application *(Not to exceed 3 pages)*	
Introduction to Supplemental Application *(Not to exceed one page)*	
A. Specific Aims	24
B. Background and Significance	25
C. Preliminary Studies/Progress Report/ Phase I Progress Report (SBIR/STTR Phase II ONLY)	31
D. Research Design and Methods	35-46
E. Human Subjects	
Protection of Human Subjects (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Women (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Minorities (Required if Item 4 on the Face Page is marked "Yes")	
Inclusion of Children (Required if Item 4 on the Face Page is marked "Yes")	
Data and Safety Monitoring Plan (Required if Item 4 on the Face Page is marked "Yes" ***and*** a Phase I, II, or III clinical trial is proposed)	
F. Vertebrate Animals	
G. Literature Cited	47
H. Consortium/Contractual Arrangements	57-58
I. Letters of Support (e.g., Consultants)	59
J. Product Development Plan (SBIR/STTR Phase II and Fast-Track ONLY)	
Checklist	60-61
Appendix *(Five collated sets. No page numbering necessary for Appendix.)*	Check if Appendix is Included
Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.	☐
Number of publications and manuscripts accepted for publication *(not to exceed 10)*	
Other items (list):	

(Items A-D: not to exceed 25 pages*)

* *SBIR/STTR Phase I: Items A-D limited to 15 pages.*

Principal Investigator/Program Director (Last, First, Middle): Eisenberg, Robert S.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/05 THROUGH 06/30/06

PERSONNEL *(Applicant organization only)* NAME	ROLE ON PROJECT	TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)* SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Eisenberg, Robert S.	Principal Investigator	12	30.0	175,700	52,710	12,545	65,255
Gillespie, Dirk	Co-PI	12	70.0	74,984	52,489	12,492	64,981
Tang, John	Asst Prof	12	100.0	72,000	72,000	17,136	89,136
SUBTOTALS →					177,199	42,173	219,372

CONSULTANT COSTS — 0

EQUIPMENT *(Itemize)*
New Axopatch Amplifier $7,500 — 7,500

SUPPLIES *(Itemize by category* Software to upgrade pClamp for Axopatch 2,000
Hardware – SCSI Interface card 500
PC desktop with Windows XP 2,500
Digidata 1322A high-speed low-noise data acquisition system 4,000
Chemicals and Reagents – Avanti Polar Lipids and salts, buffers 800
Glassware, disposable pipette tips, etc 500 — 10,300

TRAVEL Annual Biophysics Society Meetings for 3 investigators @ $1,500/each ($4,500) One additional meeting (domestic) for the PI ($1,500). One annual foreign travel for the PI to Biomade Technology ($2,500) — 8,500

PATIENT CARE COSTS — INPATIENT
OUTPATIENT

ALTERATIONS AND RENOVATIONS *(Itemize by category)*

OTHER EXPENSES *(Itemize by category)*
Membership dues and journal subscriptions to math and physical science societies 2,000
Publication costs 1,500
Photocopying costs 500 — 4,000

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		**$** 249,672
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	134,400
	FACILITIES AND ADMINISTRATIVE COSTS	39,200
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* →		**$** 423,272

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD *(from Form Page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		219,372	225,953	232,732	239,714	246,905
CONSULTANT COSTS		0	0	0	0	0
EQUIPMENT		7,500	5,000	5,000	0	0
SUPPLIES		10,300	4,500	5,000	4,500	4,500
TRAVEL		8,500	8,755	8,755	8,755	8,755
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		4,000	4,120	4,244	4,371	4,502
SUBTOTAL DIRECT COSTS		249,672	248,328	255,730	257,340	264,662
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	134,400	152,900	131,600	135,100	138,700
	F&A	39,200	40,300	41,500	42,600	43,700
TOTAL DIRECT COSTS		423,272	441,528	428,830	435,040	447,062

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)* **$** 2,175,732

SBIR/STTR Only Fee Requested					

SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period
(Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.) **$**

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Dr. Eisenberg will plan, organize and supervise the entire project. He will participate in the daily work in the areas of theory, simulation, and electrical measurements, in which he was worked before. He will participate in the design and evaluation of the mutant and modified proteins.

The salary requested for the principal investigator reflects the current salary limitations of $175,700 with a percent effort of 30%. His base salary exceeds that amount.

Dr. Gillespie will work chiefly on the theory and simulations which lead to design of mutant proteins, using existing models and theories of selectivity, improving them, and extending them.

Budget Justification:

Dr. Tang will make the electrical measurements of single channel recording.

Equipment Costs in years 2 and 3 are to replace old data acquisition equipment with new, more capable instruments

Salary costs are incremented 3% in later years according to institutional policy.

Principal Investigator/Program Director (Last, First, Middle): Eisenberg, Robert S.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/05 THROUGH 06/30/06

PERSONNEL *(Applicant organization only)*		TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)*		
NAME	ROLE ON PROJECT				SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Meijberg, Wim	Co-Principal Investigator	12	25%	0	0	0	0
Miedema, Henk	Senior Researcher	12	80%	0	0	0	0
Vrouenraets, Maarten	Reseach Scientist	12	100%	50,000	50,000	10,000	60,000
Technician		12	50%	35,000	17,500	3,500	21,000
SUBTOTALS →					67,500	13,500	81,000

CONSULTANT COSTS

EQUIPMENT *(Itemize)*
External low-pass filter and accessories — 10,000

SUPPLIES *(Itemize by category)*

Lab consumables (buffers, other chemicals, labware, etc)	23,000	Molecular Biology kits	3,000
		Restriction enzymes	3,000
Protein purification columns	3,000	Electrophysiology software upgrade	1,000
Planar Lipid Bilayer chambers and cuvets	1,000		

34,000

TRAVEL Annual Biophysics Meeting in the U.S. for the PI and Co-PI 5,600
Additional Meeting for PI to Rush University Medical Center 2,800
8,400

PATIENT CARE COSTS — INPATIENT
OUTPATIENT

ALTERATIONS AND RENOVATIONS *(Itemize by category)*

OTHER EXPENSES *(Itemize by category)*
Publication costs — 1,000

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD **$** 134,400

CONSORTIUM/CONTRACTUAL COSTS
DIRECT COSTS
FACILITIES AND ADMINISTRATIVE COSTS 39,200

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* → **$** 173,600

SBIR/STTR Only: FEE REQUESTED

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*	81,000	84,200	87,600	91,100	94,700
CONSULTANT COSTS	0	0	0	0	0
EQUIPMENT	10,000	25,000	0	0	0
SUPPLIES	34,000	34,000	34,000	34,000	34,000
TRAVEL	8,400	8,700	9,000	9,000	9,000
PATIENT CARE COSTS – INPATIENT					
PATIENT CARE COSTS – OUTPATIENT					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	1,000	1,000	1,000	1,000	1,000
SUBTOTAL DIRECT COSTS	134,400	152,900	131,600	135,100	138,700
CONSORTIUM/ CONTRACTUAL COSTS – DIRECT					
CONSORTIUM/ CONTRACTUAL COSTS – F&A	39,200	40,300	41,500	42,600	43,700
TOTAL DIRECT COSTS	173,600	193,200	173,100	177,700	182,400

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)* **$** 900,000

SBIR/STTR Only Fee Requested					

SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period
(Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.) **$**

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Justification for Foreign Support

Dr. Eisenberg has had a long standing productive collaboration with BioMade supported by awards from the Netherlands and United States Government. The design and construction of mutant and modified proteins requires substantial supporting equpment and services not available at Rush University (unfortunately) but available to BioMade through its own resources and its rental arrangements with Groningen University. The cost of such work is much less than if performed in the USA (no need for salary support for PI and Co-PI). Modern communication and long standing personal relations make it easy to work at different institutions, whether in the USA or abroad. Dr. Tang and Dr. Miedema have worked side by side for many years now and are used to exchanging ideas, tricks, and preparations of protein. Salary costs are incremented 4% in later years according to Biomade Technology policy. The fringe benefit rate is 20%. Equipment requested in 2nd year is for the purchase of an Automated protein purification set-up (FPLC) at an acqusition cost of $25,000.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Bob (*aka* Robert S) Eisenberg	Bard Professor and Chairman, Dept of Molecular Biophysics and Physiology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Harvard College, Cambridge USA	AB (summa)	1962	Biochemical Sciences
University of London, London, UK	Ph.D.	1965	Biophysics

A. Positions and Honors. Associate, 1965-1968. Department of Physiology, Duke University, Chairman: D. Tosteson. Post-doctoral fellow of P. Horowicz, along with P. Gage, C. Armstrong, etc.

Assistant Professor to Professor of Biomathematics and Physiology, 1968-76, University of California at Los Angeles.

Chairman of Physiology, 1976- …. Chairman of Department Molecular Biophysics and Physiology, Rush Medical College, Chicago, Illinois. Awarded the Endowed Chair "The Francis and Catherine Bard Professor"

Visiting Scientist, 1991-1995. Department of Physics, Brookhaven National Laboratory, NY.

Visiting Professor, 2000-2003 Computational Electronics, Univ of Illinois, Urbana-Champaign

Honors

Editorial Board, Journal of General Physiology, 1970-1991; **Editorial Board**, Journal of Computational Electronics, 2001-…; **Visiting Fellow,** Corpus Christi College, University of Cambridge (UK); **Schlumberger Medal, Physical Chemistry**; **Member Executive Board,** American Physical Society (2002- …)

Chairman of Symposium. **Nerve Impulse: From Conduction to Channels** by way of Conductance at 100th Anniversary Meeting American Physiological Society, 1987.

Chairman of Minisymposia. **Moving through (Biological) Channels** and **Ionic Movement through Biological Channel**s Society of Industrial and Applied Mathematics Conference on Applied Probability in Science and Engineering, New Orleans, 1990.

Organizer of Workshop: **From Structure to Permeation in Open Ionic Channels**. Biophysical Society Annual Meeting, Washington D.C., 1993

Chairman of Symposium: **Ionic Channels Natural Nanotubes** American Physical Society, 2000.

Chairman and Organizer of Novartis Meeting: **Physical Models of Ion Permeation**, 2000

Chairman and Organizer of Symposium International Conference Computational Nanoscience,: **Nanostructure Simulation from thin oxides to biological ion channels**. 2001

Co-organizer of Yangtze Conference on Fluids and Interfaces Chairman, **Ion Channels Session**

Organizer, Chairman **Nanostructures: biological ion channels to thin oxides.** Nanotech 2003

Co-organizer, Chair **Physical Models of Ion/Protein Interactions** Amer Physical Society 2003

Chairman **Physics of Ion Interactions with Proteins** American Physical Society 2004

Selected Papers and Contributions. *Co-designer* of Axopatch Amplifier(s) sold by Axon Instruments, for patch clamp recording. ***Co-designer* Perfusing Pipettes**, a hardware kit for perfusing patch pipettes.

Co-author **PNP-Online** Interactive software for running Poisson Nernst Planck theory.
Patent Application, with BioMade Corp. Liquid Based Electronic Device
Barcilon, V., Cole, J. and Eisenberg, R.S. (1971) A singular perturbation analysis of induced electric fields in nerve cells. SIAM J. Appl. Math. 21: No. 2, 339-354.
Eisenberg, R., Barcilon, V., and Mathias, R (1979) Electrical properties of spherical syncytia. Biophys. J. 25: 151-180 (1979).
K.E. Cooper, P.Y. Gates, and Eisenberg, R.S. (1988). Diffusion theory and discrete rate constants in ion permeation. J. Membrane Biol. 106: 95-105.
Tang, J, Wang, J, F. Quandt, R Eisenberg. (1990) Perfusing pipettes. Pflügers Arch. 416:347-350.
Eisenberg, R.S. (1990) Channels as Enzymes. J. Memb. Biol., 115, 1-12
Barcilon, V., D.P. Chen, and R.S. Eisenberg. (1992) Ion flow through narrow membrane channels. Part II. SIAM Journal of Applied Mathematics 52:1405-1425.
Wang, J., Tang, J.M., and R.S. Eisenberg (1992). A calcium conducting channel akin to a calcium pump. J. Membrane Biology 130:163-181.
Eisenberg, R.S., Klosek, M.M., and Schuss, Z. (1995) Diffusion as a chemical reaction: stochastic trajectories between fixed concentrations. J. Chem. Phys., 102(4): 1767-1780 (1995)
Eisenberg, R.S. (1996) Computing the field in proteins and channels. J. Membrane Biol. 150:1-25
Hollerbach, U., Chen, D.P., Busath, D. D., and Eisenberg, B. (2000) Predicting function from structure using the Poisson-Nernst-Planck equations: gramicidin A channel. Langmuir 16:5509-5514.
Nonner, W., L. Catacuzzeno, and Eisenberg, B. (2000). Binding and Selectivity in L-type Ca Channels: a Mean Spherical Approximation. Biophysical Journal 79: 1976-1992.
Nonner, W., L. Catacuzzeno, and Eisenberg, B. (2000). Binding and Selectivity in L-type Ca Channels: a Mean Spherical Approximation. Biophysical Journal 79: 1976-1992.
Nonner, W., Gillespie, D., Henderson, D., and Eisenberg, Bob. (2001) Ion accumulation in a biological calcium channel: effects of solvent and confining pressure. J Physical Chemistry *B* 105: 6427-6436
Schuss, Zeev, Nadler, Boaz, and Eisenberg, R.S. (2001) Derivation of PNP Equations in Bath and Channel from a Molecular Model, Phys Rev E 64: 036116 1-14)
Gillespie, Dirk, Nonner, W., Henderson, Douglas and Eisenberg, Robert S. (2002) A physical mechanism for large-ion selectivity of ion channels. Physical Chemistry Chemical Physics. 4, 4763-4769
Gillespie, Dirk, Nonner, W., and Eisenberg, Robert S. (2002) Coupling Poisson-Nernst-Planck and Density Functional Theory to Calculate Flux. Journal of Physics (Condensed Matter) 14: 12129–12145.
Gillespie, Dirk, and Eisenberg, Robert S. (2002). Physical descriptions of experimental selectivity measurements in ion channels. European Biophysics Journal 31: 454-466).
Boda, D, Busath, D, Eisenberg, B, Henderson, D, and Nonner, W (2002) Monte Carlo Simulations, ion selectivity in Na channel, charge-space competition Physical Chem Chem Physics 4 5154-5160.
Eisenberg, Bob (2003) Proteins, Channels, and Crowded Ions Biophysical Chemistry 100: 507 - 517.
Wigger-Aboud, S., Saraniti, M. and R. Eisenberg. (2003) Self-consistent particle based simulations of three dimensional ionic solutions. Nanotech 3: 443
Gillespie, Dirk, Nonner, Wolfgang, and Eisenberg, Robert S. (2003) Density functional theory of charged, hard-sphere fluids. Phys Rev E 68 0313503 1-10 (2003)
Nadler, Boaz, Hollerbach, Uwe, Eisenberg, Bob. (2003) The Dielectric Boundary Force and its Crucial Role in Gramicidin. Phys. Rev. E 68 021905 p1-9.

Boda, Dezso, Gillespie, Dirk, Nonner, Wolfgang, Henderson, Douglas and Bob Eisenberg. (2004) Computing induced charges in inhomogeneous dielectric media: application in a Monte Carlo simulation of complex ionic systems Phys Rev E 69, 046702.

Other Support:

Current:

1. DARPA Contract ends Dec. 13, 2004 and is for the construction of a bilayers setup using standard methods of silicon fabrication.
Grant number: BAA-01-007 F30602-01-2-0584 "SimBiosys: Moving to Moldice".
R.S. Eisenberg, P.I.

2. NIH research grant support work of Zeev Schuss (and his students) and Wolfgang Nonner (and his students, at the University of Miami, through a large subcontract) to work on models of permeation, gating, and gating current in ion channels.
Grant number NIH USPHD 1 RO1 GM 67241-02 "Mathematics of Ions in Protein Channels". R.S. Eisenberg, P.I.

3. BSF: United States – Israel Bi-National Foundation "Computational Models of Ionic Channels/Biol. Membranes". R.S. Eisenberg co-P.I.

Support completed in the last three years:

BSF: United States – Israel Bi-National Foundation "Non-Equilibrium Statistics of Open Ionic Channels". R.S. Eisenberg, P.I. Support ended 9/30/02.

DARPA: BAA 00-09-014 "Detection of Environmental Bioagents by Ion Channels". R.S. Eisenberg, P.I. Contract ended 12/31/02.

NSF INT – 0221738 Travel award for 12 U.S. scientists to participate in the "Interdisciplinary Workshop Introducing Physicists and Chemists to Ions in Protein Channels". The workshop was held in Nanjing China Oct. 12-18, 2002. R.S.Eisenberg, P.I.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Dirk Gillespie	Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Johns Hopkins University	BA	1994	mathematics
Northwestern University	MSc	1996	mathematics
Rush University	PhD	1999	physiology
University of Texas at Austin		1999-2000	mathematics
University of Miami School of Medicine		2000-2003	biophysics

NOTE: The Biographical Sketch may not exceed four pages. Items A and B, together, may not exceed two of the four-page limit.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

Positions

Rush University (Rush-Presbyterian-St. Luke's Medical Center), Dept. of Molecular Biophysics and Physiology: Instructor (1998 –1999), Visiting Instructor (1999 – 2003)

University of Texas at Austin, Department of Mathematics: Visiting Scholar (October 1999 – January 2000), Lecturer (January 2000 – May 2000)

University of Miami School of Medicine, Department of Biophysics and Physiology. Non-Enrolled (Postdoctoral) Fellow (June 2000 – May 2003), Voluntary Assistant Professor (June 2003 – present)

Rush University Medical Center, Dept. of Molecular Biophysics and Physiology: Assistant Professor (June 2003 – present)

Honors

Johns Hopkins University: Department of Mathematics Honors (1994), General Honors (1994), two-time winner William S. Todman Scholarship for academic excellence (1992 and 1993)

Society for Industrial and Applied Mathematics (SIAM): winner SIAM Student Paper Prize (1999)

Rush University: Carlson Luckhardt Endowed Scholarship for academic excellence (1998)

Co-organizer, Physical Models of Ion/Protein Interactions, American Physical Society March meeting (2003)

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

D. Gillespie and R. S. Eisenberg, Modified Donnan potentials for ion transport through biological ion channels, Physical Review E 63, 061902 (2001).

W. Nonner, D. Gillespie, D. Henderson, and B. Eisenberg, Ion accumulation in a biological calcium channel: Effects of solvent and confining pressure, Journal of Physical Chemistry B 105, 6427-6436 (2001).

D. Gillespie and R. S. Eisenberg, Physical descriptions of experimental selectivity measurements in ion channels, European Biophysics Journal 31, 454-466 (2002).
D. Gillespie, W. Nonner, and R. S. Eisenberg, A physical model for large-ion selectivity of ion channels, Physical Chemistry Chemical Physics 4, 4763-4769 (2002).
D. Gillespie, W. Nonner, and R. S. Eisenberg, Coupling density functional theory and Poisson-Nernst-Planck theory to calculate ion flux, Journal of Physics: Condensed Matter 14, 12129-12146 (2002).
D. Gillespie, W. Nonner, and R. S. Eisenberg, Density functional theory of charged, hard sphere fluids, Physical Review E 68, 031503 (2003).
D. Boda, T. Varga, D. Henderson, D. D. Busath, W. Nonner, D. Gillespie, and B. Eisenberg, Monte Carlo simulation study of a system with a dielectric boundary: Application to calcium channel selectivity, Molecular Simulation 30, 89-96 (2004).
D. Boda, D. Gillespie, W. Nonner, D. Henderson, and R. S. Eisenberg, Computing induced charges in inhomogeneous dielectric media: Application in a Monte Carlo simulation of complex ionic systems, Physical Review E 69, 046702 (2004).
H. Miedema, A. Meter-Arkema, J. Wierenga, J. Tang, B. Eisenberg, W. Nonner, H. Hektor, D. Gillespie, and W. Meijberg, Permeation properties of an engineered bacterial OmpF porin containing the EEEE-locus of Ca2+ channels, Biophysical Journal in press (2004).
W. Nonner, A. Peyser, D. Gillespie, and B. Eisenberg, Relating microscopic charge movement to macroscopic currents: The Ramo-Shockley theorem applied to ion channels, Biophysical Journal in press (2004).

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and responsibilities of principal investigator identified above.

co-PI Collaborative Linkage Grant (NATO Science Programme, Cooperative Science and Technology Sub-Programme) 2004-2005. Computer Simulation Studies of the Selectivity and Permeation of Ions in Physiological Channels. Goal: Use Monte Carlo, density functional (DFT), MSA, and SPM simulations of channels to understand physics of binding and selectivity of ions in highly-selective ion channels. Dr. Gillespie is using the DFT, MSA, and SPM models to study the selectivity. These are the same models to be extended through this proposal. In conjunction with Wolfgang Nonner (University of Miami School of Medicine), Dr. Gillespie developed these models while a postdoc with Prof. Nonner (2000-2003).

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Meijberg, Wim	Chief Scientific Officer at Biomade Technology Foundation

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Groningen, Groningen, The Netherlands	Masters	1987-1992	Chemistry
University of Groningen, Groningen, The Netherlands	Ph. D	1993-1998	Biochemistry/Biophysics
Imperial College of Science, Medicine and Technology, London, UK		1998-2000	Biophysics

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

Positions:

2000-2004 Project leader, Biomade Technology, Groningen, The Netherlands
2003- Coordinator for the biocomponent 'BioNanoSystems' of the National Nanotechnology Project in the Netherlands 'Nanoned'
2004- Chief Scientific Officer , Biomade Technology, Groningen, The Netherlands

Honors:

1998 Training and Mobility of Researchers Marie Curie Research Training Grant by the European Commission
1998 Long Term Postdoctoral Fellowship by the European Molecular Biology Organisation.
2000 Joint winner 'Max Gruber prize' for the best paper in the Life Sciences from the University of Groningen

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

1. Meijberg W, Schuurman-Wolters GK, Robillard GT. Interdomain interactions between the hydrophilic domains of the mannitol transporter of Escherichia coli in the unphosphorylated and phosphorylated states. Biochemistry 1996 Feb 27;35(8):2759-66
2. Vanderheeren G, Hanssens I, Meijberg W, Van Aerschot A. Thermodynamic characterization of the partially unfolded state of Ca(2+)-loaded bovine alpha-lactalbumin: evidence that partial unfolding can precede Ca2+ release. Biochemistry 1996 Dec 24;35(51):16753-9
3. Van Nuland NA, Meijberg W, Warner J, Forge V, Scheek RM, Robillard GT, Dobson CM. Slow cooperative folding of a small globular protein HPr. Biochemistry 1998 Jan 13;37(2):622-37
4. Meijberg W, Schuurman-Wolters GK, Robillard GT. Thermodynamic evidence for conformational coupling between the B and C domains of the mannitol transporter of escherichia coli, enzyme II^{mtl}. J Biol Chem 1998 Apr 3;273(14):7949-56
5. Meijberg W, Schuurman-Wolters GK, Boer H, Scheek RM, Robillard GT. The thermal stability and domain interactions of the mannitol permease of Escherichia coli. A differential scanning calorimetry study. J Biol Chem 1998 Aug 14;273(33):20785-94.

6. Robillard GT, Meijberg W, Schuurman-Wolters G. Mechanistic aspects of energy coupling in the Escherichia coli mannitol phosphotransferase system: a domain approach. Biochem Soc Trans 1998 Aug;26(3):532-8.
7. Creveld LD, Meijberg W, Berendsen HJ, Pepermans HA. DSC studies of Fusarium solani pisi cutinase: consequences for stability in the presence of surfactants. Biophys Chem 2001 Aug 30;92(1-2):65-75
8. Booth PJ, Curran AR, Templer RH, Lu H, Meijberg W. Manipulating the folding of membrane proteins: using the bilayer to our advantage. Biochem Soc Symp 2001;(68):27-33
9. Booth PJ, Templer RH, Meijberg W, Allen SJ, Curran AR, Lorch M. In vitro studies of membrane protein folding. Crit Rev Biochem Mol Biol 2001;36(6):501-603
10. Meijberg, W, Booth, PJ. The activation energy for insertion of transmembrane □-helices is dependent on membrane composition. J. Mol. Biol. 2002; 319(3):839-853
11. Allen, S.J., Curran,A.R., Templer, R.H., Meijberg, W., Booth, P.J. Folding Kinetics of an α Helical Membrane Protein in Phospholipid Bilayer Vesicles. J. Mol Biol 2004; 342(4), Sept 24: 1279-1291
12. Allen, S.J., Curran,A.R., Templer, R.H., Meijberg, W., Booth, P.J. Controlling the Folding Efficiency of an Integral Membrane Protein J. Mol Biol 2004; 342(4), Sept 24: 1293-1304
13. Miedema, H., Meter-Arkema, A., Wierenga, J., Tang, J., Eisenberg B., Nonner, W., Hektor, H., Gillespie, D., Meijberg, W. Permeation properties of an engineered bacterial OmpF porin containing the EEEE-locus of Ca-Channels. Biophys. J. 2004; in the press

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

Ongoing research support

Nanoned Cluster: Addressable delivery systems cluster leader: Meijberg
01 Sept 2004 – 01 Sept 2007,
project title 'Functional protein channels'
Role: PI

NanoNed cluster: Single molecules at interfaces cluster leader: Zuilhof
01 Sept 2004 – 01 Sept 2007
NanoNed
Project title: 'Transmembrane response systems'
Role: PI

Completed research support:

DARPA: BAA 00-09-014 "Detection of Environmental Bioagents by Ion Channels" R.S. Eisenberg, P.I. Contract ended 12/31/02.

ERBFMBICT_972625 PI: Booth
01 Mar 1998 – 28 Feb 2000
European Commision
Project title: 'The folding of membrane proteins: influence of lipid lateral pressure'
Role: Grant holder and co-investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Miedema, Henk	Senior Researcher at Biomade Technology Foundation

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Groningen, The Netherlands	1987	Masters	Plant Physiology
University of Groningen, The Netherlands	1992	Ph.D.	Electrophysiology
Vrije University Amsterdam, The Netherlands	1992-1994		Electrophysiology
McGill University, Montreal	1994-1994		Electrophysiology
Pennsylvania State University, PA	1995-1997		Electrophysiology

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

D. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

1997 - 1999 Research Associate, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico.
1999 - 2001 Research Associate, Dept. of Plant Sciences, University of Cambridge, UK.
2001 - 2002 Assistant Professor, Facultad de Ciencias, UAEM, Cuernavaca, Mexico.
2002 - Senior Researcher, Biomade Technology Foundation, Groningen, The Netherlands.

E. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

1. Miedema, H. and Prins, H.B.A. (1991) pH-Dependent proton permeability of the plasma membrane is a regulating mechanism of polar transport through the submerged leaves of *Potamogeton lucens*. *Can. J. of Bot*., 69, 1116-1122.
2. Miedema, H., Felle, H. and Prins, H.B.A. (1992) Effect of high pH on the membrane potential and conductance of *Elodea densa*. *J. Membrane Biol*., 128, 63-69.
3. Miedema, H. and Prins, H.B.A. (1992) Coupling of proton fluxes in the polar leaves of *Potamogeton lucens* L. *J. Exp. Bot*., 43, 907-914.
4. Miedema, H. and Prins, H.B.A. (1993) Simulation of the light-induced oscillations of the membrane potential in *Potamogeton* leaf cells. *J. Membrane Biol.*, 133, 107-117.
5. Miedema, H., Walraven, van S. and Boer de A.H. (1994) Potassium selective and venturicidin sensitive conductances of F_o purified from bovine heart mitochondria, reconstituted in planar lipid bilayers. *Bioch. Biophys. Res. Com.*, 203, 1005-1012.
6. Miedema, H. and Assmann, S.M. (1996) A membrane-delimited effect of internal pH on the K^+ outward rectifier of *Vicia faba* guard cells. *J. Membrane Biol.*, 154, 227-237.
7. Miedema, H., Staal, M. and Prins, H.B.A. (1996) pH-Induced proton permeability changes of plasma membrane vesicles. *J. Membrane Biol*., 152, 159-167.
8. Miedema, H. (1997) The study of (plant) ion channels reconstituted in planar lipid bilayers. *In*: Signal transduction single cell techniques. Springer-Verlag, Heidelberg. pg. 135-152.
9. Miedema, H. and Assmann, S.M. (1998) The calculation of intracellular ion concentrations and membrane potential from cell-attached and excised patch measurement. *J. Membrane Biol.*, 166, 101-110.

10. Romano, L.A., Miedema, H. and Assmann, S.M. (1998) Ca^{2+}-permeable, outwardly-rectifying K^+ channels in mesophyll cells of *Arabidopsis* thaliana. *Plant Cell Physiol.*, 39, 1133-1144.
11. Miedema, H., Henriksen, G.H. and Assmann, S.M. (1999) A laser microsurgical method of cell wall removal allows detection of large conductance ion channels in the guard cell plasma membrane. *Protoplasma*, 209, 58-67.
12. Miedema, H., Balderas, E. and Pantoja, O. (2000) Current oscillations under voltage clamp conditions: an interplay of series resistance and negative slope conductance. *J. Membrane Biol*., 173, 31-37.
13. Miedema, H., Romano, L.A., and Assmann, S.M. (2000) Kinetic analysis of the K^+ selective outward rectifier in *Arabidopsis* mesophyll cells: a comparison with other plant species. *Plant Cell Physiol.*, 42, 209-217.
14. Miedema, H., Bothwell, J. H. F., Brownlee, C. and Davies, J. M. (2001) Calcium uptake by plant cells - channels and pumps acting in concert. *Trends Plant Sciences*, 6, 514-519.
15. Miedema, H. and Pantoja O. (2001) Anion modulation of the slowly activating vacuolar channel. *J. Membrane Biol.*, 183, 137-145.
16. Baker, A., Northrop, F.D., Miedema, H., Devine, G.R., Davies, J.M. (2002) The non-steroidal anti-flammetory drug niflumic acid inhibits *Candida albicans* growth. *Mycopathologia*, 153, 25-28.
17. Miedema, H. **(**2002) Surface potentials and the calculated selectivity of ion channels. *Biophys. J.*, 82, 156-159.
18. Miedema H, de Boer, A.H., Pantoja, O. (2003) The gating kinetics of the slow vacuolar channel. A novel mechanism for SV channel functioning? *J. Membrane Biol.*, 194, 11-20.
19. Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa, S, Brownlee C, Jones, JD, Davies, JM, Dolan, L. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature*, 422, 442-446.
20. Miedema H., Meter-Arkema A, Wierenga J, Tang J, Eisenberg B, Nonner W, Hektor, H, Gillespie, D, Meijberg, W. (2004) Permeation properties of an engineered bacterial OmpF porin containing the EEEE-locus of Ca-channels. *Biophys. J.*, in the press.

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
John M. Tang	Assistant Professor Department of Molecular Biophysics and Physiology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Charleston, West Virginia	B.S.	1973	Biology
University of Illinois at Medical Center, Chicago	M.S.	1977	Physiology
Rush University, Chicago	Ph.D.	1991	Physiology

NOTE: The Biographical Sketch may not exceed four pages. Items A and B, together, may not exceed two of the four-page limit.

C. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

Positions:

2003-Present	Department representative and member of Rush's Research and Academic Laboratory Safety Standing Committee (RALSC)
1998-Present	Assistant Professor, Department of Molecular Biophysics and Physiology, Rush Medical College, Chicago, IL.
1991-1998	Instructor, Department of Physiology, Rush Medical College, Chicago, IL.
1984-1991	Research Associate/Laboratory Supervisor, Department of Physiology, Rush Medical College, Chicago, IL.
1981-1984	Research Assistant, Department of Physiology, Rush Medical College, Chicago, IL.
1977-1978	Instructor, Medical Dietetics Department, University of Illinois at the Medical Center, Chicago, IL.
1973-1977	Research and Teaching Assistant, Department of Physiology and Department of Medical Dietetics, University of Illinois at the Medical Center, Chicago, IL.
1970-1973	Teaching Assistant, Department of Biology, University of Charleston, Charleston, W. Va.

Professional Societies: Chi Beta Phi Honorary Scientific Fraternity, Epsilon Chapter
Biophysical Society

Honors:

1992	Outstanding graduate student award. Rush University, Chicago, IL.
1985-1989	National Institutes of Health traineeship
1973-1977	Teaching assistantship with full tuition waiver. University of Illinois at the Medical Center, Chicago, IL.
1970-1973	Dean's List. University of Charleston, Charleston, W. Va.
1970-1973	Full tuition scholarship. University of Charleston, Charleston, W. Va.

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

Nelson, D. J., **Tang, J. M.** and Palmer, L. G. (1984) Single-channel recording of apical membrane chloride conductance in A6 epithelial cells. Journal of Membrane Biology. 80:81-89.

Nelson, D. J., Jacobs, E. R., **Tang, J. M.**, Zeller, J. M. and Bone, R. C. (1985) Immunoglobulin G-induced single ionic channels in human alveolar macrophage membranes. Journal of Clinical Investigation. 76:500-507.

Cooper, K. E., **Tang, J. M.**, Rae, J. L. and Eisenberg, R. S. (1986) A cation channel in frog lens epithelia responsive to pressure and calcium. Journal of Membrane Biology. 93:259-269.

Tang, J. M., Wang, J. and Eisenberg, R. S. (1989) K^+-selective channel from sarcoplasmic reticulum of split lobster muscle fibers. Journal of General Physiology. 94:261-278.

Tang, J. M., Wang, J., Quandt F. N. and Eisenberg, R. S. (1990) Perfusing pipettes. Pflügers Arch. 416:347-350.

Wang, J., **Tang, J. M.** and Eisenberg, R. S. (1992) A calcium conducting channel a kin to a calcium pump. Journal of Membrane Biology. 130:163-181.

Tang, J. M., Wang, J. and Eisenberg, R. S. (1992) Perfusing patch pipettes. In *Methods in Enzymology*. Chapter 10. Volume 207:176-181. B. Rudy and L.E. Iverson, editors. Academic Press, Florida.

Tang, J. M., Wang, J. and Eisenberg, R. S. (1992) Studies on intact sarcoplasmic reticulum: patch clamp recording and tension measurement in lobster split muscle fibers. In *Methods in Enzymology*. Chapter 48. Volume 207:692-699. B. Rudy and L.E. Iverson, editors. Academic Press, Florida.

Tang, J. M., Quandt, F. N. and Eisenberg, R. S. (1995) Perfusion of patch pipettes. In *Patch Clamp Applications and Protocols.* Chapter 5. Volume 26. A.A. Boulton, G.B. Baker and W. Walz, editors. Humana Press, New Jersey.

van der Straaten T. A., **Tang, J. M.**, Eisenberg, R. S., Ravaioli, U. and Aluru, N. (2001) Three-dimensional Continuum Simulations of Biological Ion Channels, in Technical Proceedings of the 2001 International Conference on Computational Nanoscience and Nanotechnology, Hilton Head, SC, March 19-21, 2001, pp. 39-42.

van der Straaten, T. A., Varma, S., Chiu, S. W., **Tang**, **J. M.,** Aluru, N., Eisenberg, R. S., Ravaioli, U. and Jakobsson, E. (2002) Combining Computational Chemistry and Computational Electronics to Understand Protein Ion Channels, in Technical Proceedings of the 2002 International Conference on Computational Nanoscience and Nanotechnology, San Juan, Puerto Rico, April 21-25, 2002, pp. 60-63.

Chen, D., **Tang**, **J. M.** and Eisenberg, R. S. (2002) Structure-Function Study of Porins, in Technical Proceedings of the 2002 International Conference on Computational Nanoscience and Nanotechnology, San Juan, Puerto Rico, April 21-25, 2002, pp. 64-67.

van der Straaten, T. A., **Tang**, **J. M.,** Eisenberg, R. S., Ravaioli, U. and Aluru, N. (2002) Three-dimensional Continuum Simulations of Ion Transport Through Biological Ion Channels: Effect of Charge Distribution in the Constriction Region of Porin. Journal of Computational Electronics 1: 335-340.

van der Straaten, T. A., **Tang**, **J. M.,** Ravaioli, U., Eisenberg, R. S. and Aluru, N. (2003) Simulating Ion Permeation Through the OmpF Porin Ion channel Using Three-Dimensional Drift-Diffusion Theory. Journal of Computational Electronics 2: 29-47.

Goryll, M., Wilk, S., Laws, G. M., Goodnick S., Thornton T., Saraniti, M., **Tang, J. M.** and Eisenberg, R. S. (2003) Silicon-based ion channel sensor. Superlattices & Microstructures 34 (3-6), 451-457.

Goryll, M., Wilk, S., Laws, G. M., Goodnick S., Thornton T., Saraniti, M., **Tang, J. M.** and Eisenberg, R. S. (2004) Ion Channel Sensor on a Silicon Support Mat. Res. Soc. Symp. Proc. Vol. 820, O7.2.1-5 (2004). Proceedings Title: Nanoengineered Assemblies and Advanced Micro/Nanosystems Editors (Symposium O):Jun Liu, Jeffrey T. Borenstein, Piotr Grodzinski, Luke P. Lee, Zhong Lin Wang.

Wilk, S., Goryll, M., Laws, G. M., Thornton, T. J., Goodnick, S. M., Saraniti, M., **Tang**, **J. M.** and Eisenberg, R. S. (2004) Teflon coated silicon apertures for supported lipid bilayer membranes. (Accepted by Applied Physics Letters).

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and responsibilities of principal investigator identified above.

Other Support:

Current:

1. DARPA Contract ends Dec. 13, 2004 and is for the construction of a bilayers setup using standard methods of silicon fabrication.
Grant number: BAA-01-007 F30602-01-2-0584 “SimBiosys: Moving to Moldice”.
R.S. Eisenberg, P.I.

2. NIH research grant support work of Zeev Schuss (and his students) and Wolfgang Nonner (and his students, at the University of Miami, through a large subcontract) to work on models of permeation, gating, and gating current in ion channels.
Grant number NIH USPHD 1 RO1 GM 67241-02 “Mathematics of Ions in Protein Channels”. R.S. Eisenberg, P.I.

Support completed in the last three years:

DARPA: BAA 00-09-014 “Detection of Environmental Bioagents by Ion Channels”. R.S. Eisenberg, P.I.
Contract ended 12/31/02.

Principal Investigator/Program Director (Last, first, middle): Eisenberg, Robert S.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Vrouenraets, Maarten	Researcher at Biomade Technology Foundation

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Amsterdam, The Netherlands	Masters	1993	Organic Chemistry
Vrije Universiteit, Amsterdam, The Netherlands	Ph.D.	2003	Photodynamic Therapy

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

F. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

2000 - Researcher, Biomade Technology Foundation, Groningen, The Netherlands.

G. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.

1. Vrouenraets, M.B., Visser, G.W.M., Stewart, F.A., Stigter, M., Oppelaar, H., Postmus, P.E., Snow, G.B. and van Dongen, G.A.M.S. (1999) Development of *meta*-tetrahydroxyphenylchlorin-monoclonal antibody conjugates for photoimmunotherapy. *Cancer Res.*, 59, 1505-1513.
2. Vrouenraets, M.B., Visser, G.W.M., Loup, C., Meunier, B., Stigter, M., Oppelaar, H., Stewart, F.A., Snow, G.B. and van Dongen, G.A.M.S. (2000) Targeting of a hydrophilic photosensitizer by use of internalizing monoclonal antibodies: A new possibility for use in photodynamic therapy. *Int. J. Cancer*, 88, 108-114.
3. Vrouenraets, M.B., Visser, G.W.M., Stigter, M., Oppelaar, H., Snow, G.B. and van Dongen, G.A.M.S. (2001) Targeting of aluminum (III) phthalocyanine tetrasulfonate by use of internalizing monoclonal antibodies: improved efficacy in photodynamic therapy. *Cancer Res.*, 61, 1970-1975.
4. Vrouenraets, M.B., Visser, G.W.M., Stigter, M., Oppelaar, H., Snow, G.B. and van Dongen, G.A.M.S. (2002) Comparison of aluminum (III) phthalocyanine tetrasulfonate- and *meta*-tetrahydroxyphenylchlorin-monoclonal antibody conjugates for their efficacy in photodynamic therapy *in vitro*. *Int. J. Cancer*, 98, 793-798.
5. Vrouenraets, M.B., Visser, G.W.M., Snow, G.B. and van Dongen, G.A.M.S. (2003) Basic principles, applications in oncology and improved selectivity of photodynamic therapy. *Anticancer Res.*, 23, 505-522.
6. Van Dongen, G.A.M.S., Visser, G.W.M. and Vrouenraets, M.B. (2004) Photosensitizer-antibody conjugates for detection and therapy of cancer. *Adv. Drug Deliv. Rev.*, 56, 31-52.

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

Principal Investigator/Program Director (Last, First, Middle): Eisenberg, Robert S.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Well equipped channel biophysics laboratory for reconstituting channel proteins into bilayers and recording single channel currents

Clinical: N/A

Animal: N/A

Computer:

Several adequate but aging PC setups for data acquisition and ananlysis

Office:

Standard Office Equipment

Other:

The usual

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Nothing needed beyond what is described above

Principal Investigator/Program Director (Last, First, Middle): Eisenberg, Robert S.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Biochemistry/Molecular Biology laboratory: site-directed mutagenesis and expression, production, purification of membrane proteins -Biomade, 100% available
Electrophysiology laboratory: functional characterization of channels -Biomade, 100% available
Organic chemistry laboratory: organic synthesis, chemical modification of proteins -Biomade, 100% available

Clinical: N/A

Animal: N/A

Computer:

General computing facilities: collection, storage and processing of electrophysiology data; molecular modelling of proteins - Biomade, 100% available

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

planar lipid bilayer set-up, Biomade, functional characterization of channels
Patch clamp set-up, University of Groningen, functional characterization of channels
Automated protein purification set-up, Biomade, protein purification
Fermentation facility, University of Groningen, growth of bacteria for protein production

Overall Goal: Model, simulate, construct and measure calcium binding sites in proteins.

Calcium controls an enormous range of biological functions in its role as first and second messenger. Calcium binding to ion channels directly control many biological processes. Binding is well studied in channels because channels 1) are studied one molecule at a time in hundreds of laboratories every day 2) are sensitive to ~10^{-7} M Ca^{++} 3) the physical origin of binding and movement of Ca^{++} is beginning to be understood.

Ca^{++} binding sites contain several negatively charged (acidic) amino acid residues associated with (nearly) equal numbers of mobile positive charges nearby, at large number density ('concentration') because of the 'principle of electroneutrality': in physiological solutions, the 'active site' of the L-type Ca^{++} channel includes 4 glutamates containing 4 fixed negative charges, along with 2 mobile Ca^{++} in a cylinder 3Å long × 7Å diameter, giving a number density of mobile charge ~10^{22} cm^{-3} = 30 **M**olar. (Liquid water is ~55 **M**olar.)

The free energy of crowded configurations of charge can be calculated using modern theories and Monte Carlo simulations of highly concentrated solutions, recently developed and tested by many physical chemists and now applied to binding sites of channel proteins. These models calculate free energy per mole ('activity') of bulk ionic solutions from infinite dilution, to saturation, even in ionic melts, with ~1% accuracy. We think these models allow model based engineering of proteins for a specific function, namely Ca^{++} selectivity.

Engineering of proteins for a specific function is an enormously important goal of computational biology. Here we propose to engineer a calcium binding site, using a rational, albeit simplified physical model of selectivity for design. We propose to use molecular genetics and organic chemistry to build sites; and we propose to study the sites by measuring current through a channel protein containing the designed site.

Specific Aims

1) Using models of concentrated salt solutions well-established in modern physical chemistry, we will design and mutate a nearly nonselective bacterial protein porin *(ompF)* into a Ca^{++} selective protein like the L-type calcium channel of heart. Models of binding will be based on the mean spherical approximation **MSA**, solvent primitive model **SPM,** and Monte Carlo **MC** simulations. Density functional theory (**DFT**) combined with diffusion equations will be used to compute ion flux. These theories are already known to describe natural L-type calcium channels quite well. With suitable changes, they also offer insight into Na^{+} channel of nerve and anion channels of epithelia.
2) We will improve models of selectivity (i.e., models of concentrated inhomogeneous ionic solutions)
   (a) by including better models of water
   (b) by including the important dielectric boundary force **DBF** arising from polarization charge at interfaces.
3) We will construct and test mutant proteins with the high densities of carboxyl groups and the small pore volumes needed to produce physiological Ca^{++} selectivity, using conventional site-directed mutagenesis. Amino acid(s) in the natural protein will be replaced with other amino acids with different size and chemical properties by modifying the genetic code from which the protein is made. According to our models and simulations, selectivity requires very crowded charge, as found in many natural proteins, and so we will construct and test mutants with pores of small volume, ~200 $Å^3$. Thus, mutations introducing amino acids with bulky side chains should reduce pore volume enough to produce biological selectivity of Ca^{++} *vs.* Na^{+}. Binding will be assayed by measurements of currents from single channels under a wide range of conditions, determined by the concentration of 'agonist' and electrical potential across the channel.
4) We will also make chemical modifications of amino acids in the *ompF* protein using non-genetic methods to introduce new chemical groups into *ompF* mutants. We will covalently link bulky chemical groups to amino acids of the protein, using well known methods of organic and bio- chemistry. We introduce the natural amino acid cysteine that contains sulfhydryl groups to which bulky groups can be bound and link MTSEA or MIANS (defined in Fig. 6) to them. These bulky groups will produce the crowded charge in narrow pores (found in biological channels) needed to make highly selective channels.

Background and **Significance**

Background for Specific Aim #1 & 2: Models of Concentrated Salt Solutions and Selectivity

Summary of Background and Significance. The fundamental property underlying selectivity in our models is shown below. Selectivity arises from the competition between crowded charge effects and the electric field, modulated significantly by the dielectric boundary force. In these models, the precise coordinates of atoms are not involved, nor are chemical bonds in the sense of covalent bonds created by orbital delocalization.

Selectivity Arises in a Crowded Space

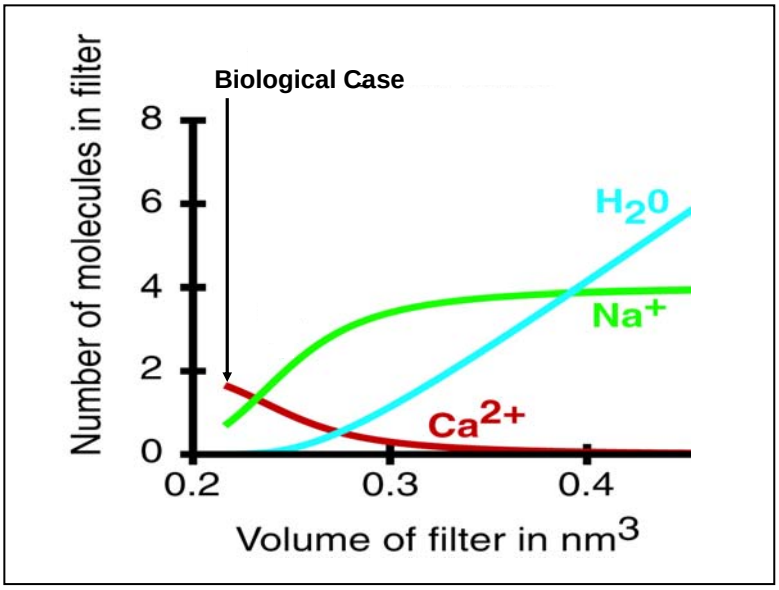


Fig. 1 Selectivity in L-type Ca channel. The biological case has 0.1 M NaCl and 1 μM $CaCl_2$ in the baths. As the volume of the selectivity filter decreases, water is excluded from the filter by crowded charge effects and Ca^{++} enters the filter and displaces Na^+.

Significance: An Essay on Selectivity in Channels. The following paragraphs offer a general introduction to selectivity and are a somewhat reworked version of [55]. Specific discussion of earlier rate models of permeation and selectivity can be found in [54] and [37]. I apologize for overlap with parts of the Research Design and Background Sections of this proposal. Each Section is meant to be readable by itself.

Ion channels are proteins with holes down their middle and have enormous biological and medical importance [3; 8; 94; 151; 175]. Ion channels are responsible for signaling in the nervous system; coordination of muscle contraction, including the coordination of cardiac muscle that allows the heart to function as a pump. Ionic channels modulate and control transport in the kidney, gastrointestinal tract, indeed in all epithelia. They modulate and control the secretion of hormones. Indeed, few properties of cells and organs are not controlled by channels. As measurements of channel function are made in *intra*-cellular organelles [191; 193; 210] ion channels are being found to control a wide range of the function of these structures, which are central to nearly all life. How many subcellular processes are directly controlled by channels is not yet known, and their role in the transport of proteins inside cells (in the endoplasmic or sarcoplasmic reticulum, Golgi apparatus, etc.) is just emerging and somewhat controversial, although signs of that role were found long ago [191; 193; 210].

The widespread role of ion channels means they are an inviting target for drugs. A substantial fraction of drugs used by physicians act on channels. The widespread role of channels also means that disease occurs when channels malfunction. A large and rapidly increasing number of diseases called channelopathies are being investigated clinically, as a Google search on that word shows.

Channels are very important biologically and medically but they are much less complex than many other living systems of comparable importance. Most channel function occurs in one protein. Even when several other proteins regulate channel function, the pore of the channel (through which ions move) is almost always in just one protein molecule itself. The physics of ion movement through channels, once they are open, is simple: only electrodiffusion, convection and heat flow occur in condensed phases under biological conditions, and electrodiffusion clearly dominates the properties of permeation through open channels in many cases. The challenge is to **predict the current of ions** of different types and concentrations through many different types of channels **using only the language of physics of condensed phases**. Enormous opportunities for good science exist because of the huge literature of channels. Thousands of scientists (literally) measure the ions going through channels, one molecule at a time, every day. Some two thousand papers on channels are presented annually at the meeting of the American Biophysical Society, and some 10,000 amplifiers, suitable only for measuring currents through single channels, have been sold in the last decade or two.

Ions and channels are inseparable. Without ions, channels do not function. Ions and proteins are also inseparable. The fixed charge of the protein demands counter-ions in a nearby ionic atmosphere: otherwise, impossible electrical forces are created, see first paragraphs of Feynman [64]. Cohn and Edsall recognized long ago that proteins bristle with charge (cf. Cohn, as cited on p. 73 of [178]) and thus with counter ions.

We shall argue that many of the properties of channels are produced by the crowding of counterions near active sites, allowing their different sizes and charges to determine and modulate the free energy available for ion permeation. We argue that similar energetics—of crowded charge produced by the electric field of active sites—are likely to be important in protein function in general [56]. “Channels are enzymes” [56] is a working hypothesis likely to be helpful in the design of productive experiments on both channels and enzymes. Of course, some proteins (and enzymes in particular) are likely to use additional sources of energy beyond those in the simplest physical models of ion channel selectivity.

The fixed charge that crowds ions is permanent in the sense that it arises from the nature of the chemical bonds of the protein—i.e., from the solution of Schrödinger’s equation. Those bonds are built into proteins following the blueprint of their DNA. The permanent charge of proteins and the surrounding ionic atmosphere is under genetic, and thus evolutionary control. The modulation of the energy of crowded charge is a mechanism by which the genome and protein could control biological function.

The ionic atmosphere surrounding an active site can be viewed as a biologically important structure of varying size. Large amounts of energy are involved in changing the charge and number density of the ionic atmosphere near active sites, even if the water and solution itself are (nearly) incompressible. In crowded solutions, the changes in the energy of the ionic atmosphere are large and have substantial effects, as has been shown in physical chemistry in the last decade or two. [9; 10; 38; 50; 181-185]. These properties of the ionic atmosphere resemble the properties of dense plasmas in a dielectric background whether the plasma is that of a real gas [28; 99; 100] or that of a gas of quasi-particles, holes and ‘electrons’ in a semiconductor [47; 88-90; 95; 103; 122; 179] and the resemblance can be exploited to make calculations of ionic properties using software of semiconductor physics [1; 202].

Physical Chemistry of Concentrated Solutions. Many specific chemical properties of concentrated salt solutions (having nothing to do with proteins) are known now to arise from the excess free energy of crowded spheres. In modern physical chemistry [9; 10; 38; 50; 181-185], the diameter and charge of ions alone is

enough to explain many complex properties of concentrated salt solutions without considering other specific chemical properties of the ionic species, e.g., without considering changes in orbital structure. The energy in the physical effects of crowded charge is so large that the energies coming from changes in orbital structure are not important in determining the fundamental physical property of solutions, the free energy per mole. Specific chemical interactions with the solvent (produced by electron delocalization, for example) are not included in these modern successful treatments of concentrated salt solutions because the energies are small compared to the energies of crowded charge. Polarization effects are included, but an implicit treatment, with the limited resolution of a dielectric constant is enough to predict the fundamental property—the free energy per mole—from infinite dilution to saturation.

Models that analyze the free energy of crowded spheres, treating the water as a uniform dielectric, with dielectric constant of the ionic solution (not of pure water), do remarkably well because the dominant effect in concentrated salt solutions comes from the electrostatic energy of crowded spheres and the entropy necessary to crowd (uncharged) spheres together. The centers of crowded spheres can only approach within 1 diameter of each other (more precisely, the sum of the radii of two perhaps unequal spheres) [208; 209]. Debye-Hückel theory treats its central ion as a sphere but it treats other ions as points—even though they are identical to the central ion—so their centers of charge can approach within a radius, not diameter of each other. The electric field is at its strongest in this region close to an ion so the errors in Debye-Hückel theory are large, particularly at high concentrations. For concentrated solutions, Debye-Hückel theory needs to be replaced, and the Mean Spherical Approximation **MSA** is the simplest, least accurate but often satisfactory replacement [10; 17; 19; 50; 114; 162; 181-185; 198-200; 208; 209]. We use the **MSA** of Simonin et al., [50; 181-185], expanded into a primitive model of the solvent as well, the **SPM** (suggested to us by Douglas Henderson [22; 24-26; 146; 195-197; 205; 219]. “We” here means Wolfgang Nonner, who has led this collaboration more than anyone else, with important contributions from Doug Henderson, Dirk Gillespie, Dezso Boda, and others.

The **MSA** (or its analogs) represent solvation energies only in a primitive way as the interaction of finite charged spheres (ions) with each other and with a uniform dielectric (water). Nonetheless, the theories quite successfully predict the activity coefficients of ions over a range of concentrations. The finite (i.e., excluded) volume of the ions distorts the electric field (compared to that of point particles) and accounts for much of the excess free energy; the finite volume of uncharged spheres accounts for much of the entropic component of the excess free energy of the solution. Specific chemical effects exist, but are small in comparison, fundamentally because disturbances in the electric field swamp the energetic effects of orbital delocalization under the conditions of interest here. It seems likely then that similar effects might be important in the concentrated ionic atmosphere near active sites of proteins.

Selectivity in channels. Before we knew of the recent work on concentrated salt solutions, it seemed unlikely to us that such a simple (and iconoclastic) model could describe phenomena of biological importance or complexity. We knew that excluded volume effects are important in the theory of electrolyte solutions and therefore must be present inside a channel, even if we thought they were not enough to explain complex selectivity properties. Channels contain large numbers of ions at very high density (i.e., ‘concentration’) because the walls of channels have fixed charge and the volume of the pores of channels is small. Excluded volume effects are particularly important in highly concentrated electrolyte solutions and therefore we expect them to be particularly important in ion channels.

In fact, we now know that many complex selectivity properties can be explained by a simple model of the crowded charge in and near their selectivity filter, using the same principles that are used to understand concentrated salt solutions without proteins. [21; 23; 33; 34; 55; 71; 73; 129; 140-147]. In this treatment, selectivity arises from the properties of the concentrated ion plasma near the active site, more than anything

else. The complex properties of selectivity arise from changes in the density of the concentrated ion plasma near the active site. The density of the ions of the plasma of the ionic atmosphere varies with the permanent charge of the active site; as the charge on the active site changes, the plasma density changes, because of the demand for electroneutrality. Many other properties of proteins are influenced by changes in the density of permanent charge at their active site. Crowded charge effects need to be considered (in my opinion) whenever the charge or volume of the active site changes. Crowded charge effects may be important determinants of conformation changes, drug binding, and protein folding, although that idea is certainly unproven and needs much further thought so one knows how to test and implement such ideas in the context of previous work.

Whenever the density of permanent charge at the active site changes, the density of the compressible plasma of the ionic atmosphere changes as well, and that change in density has large effects because the energy stored in the plasma is so large. The permanent charge of the active site and strength of the electric field enforce severe crowding of mobile ions, allowing their specific chemical properties (that arise chiefly from their excluded volume and charge) to become important determinants of protein function. In this view, selectivity and other properties of proteins arise from the balance of electrical and excluded volume in places crowded with charge, e.g., the selectivity filter of channels and active sites of proteins. In this view, the protein modulates and uses the energies of ions in the dense ion plasma near the active site, much as an automobile engine modulates and uses gasoline and air.

Indeed, the energetics of a compressible plasma are enough to explain complex chemical and biological properties of channels without invoking delocalization of outer electrons or a specific geometrical arrangement of atoms of the protein. In the **MSA** version of this idea, the energy of the protein is (nearly) ignored; the **MSA** model leaves out both specific chemical bond energies and energy stored in the protein away from the active site. The theory nonetheless is quite successful in dealing quantitatively with phenomena of selectivity, using only known properties of concentrated salt solutions and two physical parameters to describe the role of the protein, although these models will undoubtedly need successive extension.

This physical explanation of selectivity is quantitative and physically specific in contrast to verbal descriptions of selectivity as the result of hydrophilic/hydrophobic interactions at binding sites. I think utilitarian engineering analysis is likely to be more productive than narratives of trajectories or traditional literary discussions of binding sites. What is needed in my view is engineering as usual, but now engineering on the (sub) nanoscale of proteins, relying mostly on the techniques of ‘reverse engineering’ (i.e., the solution of inverse problems [76]), trying to design rather than analyze.

The physical origin of selectivity between two ions is predicted without ambiguity in physical models, as well as the dependence of that selectivity on the properties of the channel protein, namely its charge, size of its charges, volume of its selectivity filter, and dielectric coefficient. But the physical basis involves a number of terms, most of which depend on the concentration of all other ions. Thus, the selectivity depends on the (1) electrical potential, (2) ideal chemical potential (i.e., concentration), (3) entropy of (uncharged) hard spheres, and (4) energy of hard spheres. Each of these terms is different for each type of ion and most depend on concentration. Thus, to understand the selectivity between two particular situations of biological interest, one must compare all the terms. This is not hard to do, since the equations are algebraic and explicit, and all terms are known without possibility of adjustment. The terms are often of nearly the same size, however, so the results must be computed. Their relative size cannot be evaluated by verbal discussion.

Qualitative analysis is possible, on the other hand, if a few terms dominate a phenomenon of interest. Of course, quantitative analysis, as just described, is then needed to establish the validity of the qualitative analysis. Quantitative analysis makes it easier to check our working hypothesis and find where other factors come into play, beyond crowded charge. Interestingly, the most important biological property of L-type calcium

channels—their selectivity for Ca^{++} vs. Na^{+} —seems to be dominated by a few terms. This is not the only case in which evolution seems to have used a particular subset of all physical possibilities: see any text of physiology or biophysics. It seems that evolution often chooses the strategy of a sensible engineer, Keep it Simple Stupid, when it chooses the physics to create a biological function. Biological complexity often arises from the structure, not the physics of the system. One imagines that if a few terms dominate the physics of a system, genetic control is easier, and the resulting system is more likely to follow simple robust rules that survive uncontrolled changes in environment. Perhaps this is why crowded charge seems enough to explain selectivity in some Ca channels. Other factors might contribute significant free energy but **biology seems to have chosen to control the free energy of binding mostly by the energy of crowded charge**.

The selectivity between Ca^{++} and Na^{+} arises in a simple way. The four glutamates of the channel demand the presence of four mobile positive charges nearby. If only Ca^{++} is present, two Ca^{++} provide the four charge. If only Na^{+} is present, four Na+ provide the four charges, but **the four Na^{+} are twice as crowded as two Ca^{++}** because Ca^{++} has the same diameter as Na^{+}. The four Na^{+} occupy twice the space of the two Ca^{++}. The free energy necessary to pack the extra two charged spheres into the channel accounts for ~60% of selectivity. About 35% of the selectivity comes from the different electrical potential found in the channel when Ca^{++} is present. The electrical potential is different because Ca^{++} provides better screening (of the negative charge of the glutamates). The double valence of Ca^{++} allows two charges to approach within one ion diameter of the glutamate oxygen where Na^{+} allows only one. 5% of the selectivity comes from other effects, e.g., entropy resulting from specific arrangements of ions and glutamates.

It is important to emphasize how different this view of selectivity is from the traditional view. Traditional models of binding more or less ignore the electrical term altogether, shown by the absence of Coulomb's law in their derivations or by the absence of a dielectric constant or permittivity parameter in their output equations. Physical models predict electrical potentials of hundreds of millivolts—i.e., of the order of 4 k_BT/e—and those potentials vary more or less linearly with the logarithm of Ca^{++} concentration under standard conditions. Thus, a substantial fraction of selectivity in a physical model comes from an effect ignored in most traditional treatments of binding and selectivity, the electrical energy needed to bind an ion to a charged site [58].

Traditional models of binding and selectivity (and of enzyme kinetics, for that matter [49; 105]) also assume rate constants and binding constants independent of concentration of the binding species. This seems unlikely on physical grounds, because the binding of a charged group to a charged site will inevitably change the electrical potential, according to Gauss' law of electrostatics (i.e., Maxwell's equations) [58], and changing a potential barrier will change the rate constant for crossing the barrier. The change in potential will be large because binding sites are small and "capacitance to infinity" is small, i.e., self-energy is large [57; 58]. Single charge devices use such effects [74; 117] and are studied experimentally in many laboratories every day. Similar effects seem unavoidable in proteins. Other language describes the same physics: shielding effects dominate many properties of channels as well as ionic solutions [36; 83; 85; 123], plasmas of real gas [28; 99; 100], or plasmas of quasi-particles, the holes and 'electrons' in a semiconductor [47; 88-90; 95; 103; 122; 179].

When shielding is important, the binding constant changes many fold as conditions vary. Thus, many of the qualitative features of binding are produced by a variation of binding constant not permitted in traditional theories of chemical kinetics.

Despite the evident limitations, what is striking is that such simple physical models as the **MSA** and **SPM** can deal quantitatively with such a large range of selectivity phenomena. The physical effects in these models are more or less confined to the concentrated ionic plasma in the selectivity filter. The properties of that plasma are calculated using known results from the physical chemistry of concentrated salt solutions.

In view of the success of these simple models, I believe that understanding of binding and selectivity should start with known physical properties of concentrated ionic solutions and add in the complexities brought by the protein, one by one. This approach might also be useful in the study of drug docking and protein folding.

Background from Channel Biophysics. Here I present background for our general viewpoint, that selectivity arises from crowded charge (more than anything else) which, I hasten to add, is our **working hypothesis**, to be improved or replaced as we find evidence for other forces in specific situations. What is most important is that this working hypothesis is specific, based on modern physical chemistry and able to make predictions with few adjustable parameters. This working hypothesis has been converted into a specific model that fits data quite well from highly selective L-type Ca channels [23; 33; 34; 55; 71; 72; 129; 140-146], which data has not been fit by other physical models, as far as I know. Specific models of other channels can be created by adding additional physics (with few extra parameters). A crowded charge model can easily fit key data from the highly selective voltage activated Na^+ channel [21]. The model shows how mutations of the EEEE locus (of the L-type calcium channel) can change the DEKA locus (of the Na^+ channel) and produce the changes in electric field and the crowding necessary to change selectivity from Ca^{++} to Na^+. A different specific model (with essentially one extra adjustable parameter) fits data from anion selective channels quite well, including the crossover of IV curves and the lyotropic series of selectivity which have been hard to understand [73].

We are unaware of other physical models of selectivity in Ca^{++} channels. Rate models, based on gas phase chemical kinetics are not helpful in understanding the physical basis of selectivity because ideal gases are so different from ionic solutions: further discussion is in [37; 54]. Simulations of molecular dynamics [40; 41; 110; 111] do not provide reliable estimates of the components of binding free energy that are necessary to understand selectivity in physical terms.

Simulations of molecular dynamics do not yet provide estimates of physically important components of free energy because they are severely limited by computational constraints. It is difficult to compute total free energy. In fact, it is difficult to compute enough channel crossings to establish current voltage **IV** curves; it is nearly impossible to compute **IV** curves when ionic concentrations are extremely small like the 10^{-7} concentrations important for Ca^{++} channels, particularly in the presence of 100 mM Na^+. (Consider how many atoms are needed in the simulation if it contains ~100 Ca^{++} ions at 10^{-7} M and water at 55 M.) Thus, computations cannot be done with sufficient reliability under a range of conditions to estimate the components of free energy necessary to understand the physical basis of selectivity.

In fact, my collaborators and I [1; 202] have found it very difficult, although possible, to reproduce the fundamental properties of 1M bulk ionic solutions, the free energy per mole and the pair correlation function. Calculations would be much harder in 10^{-7} M Ca^{++}. (See our review [170] for a general discussion of these issues). Calibrated molecular dynamics simulations of biological solutions will surely be possible some day and we expect them to show the importance of molecular detail not yet in our theories. We look forward to such work and indeed hope to contribute to it ourselves, in a separate project to begin in the next year or so. But molecular dynamics simulations to date cannot estimate the components of free energy which provide physical understanding of selectivity.

Background from Physical Chemistry. Physical chemists have shown [10; 50; 51; 62; 82; 114; 181; 182; 184; 185] that simple models of concentrated salt solutions do surprisingly well in describing the fundamental property of ions in solution, even if these models describe ions as hard spheres of charge and describe water implicitly as a dielectric, not explicitly as molecules. In an early paper on selectivity [140], we were surprised to find that this theory could explain many (but certainly not all) of the complex properties measured from L-type calcium channels (measured over some 5 orders of concentration of divalent ions, and for many types of monovalent ions and many mutations) with only one or two adjustable parameters. (Other parameters of the

model were taken from physical measurements of ions and were not adjusted.) With appropriate modifications, a similar approach worked well for Na^+ channels [21] and anion channels [73], although the work on these channel types is certainly much less mature than that on L-type Ca channels. In later work [55; 71; 73; 129; 146], we have shown that treating water as uncharged spheres in a dielectric background with the **SPM** significantly improves results, which is hardly surprising since water is the most abundant particle species in the system and obviously should be described as a molecule if possible. (Indeed, the surprise in physical chemistry and also channel biology is that implicit ('primitive') models of water do as well as they do.)

The modern state of knowledge of ionic solutions is just now finding its way into reviews and textbooks [10; 15; 50; 51; 62; 183]. The calculation of the fundamental property of solutions from theory —the free energy per mole, i.e., activity or activity coefficient, when normalized—was a serious problem for physical chemists until recently and early calculations of activity or activity coefficient (admitted to be inadequate by early workers) have often led biologists to think the quantities themselves are suspect 'fudge factors'. Activity and activity coefficients are not fudge factors. Rather, experimental measurements of activity and activity coefficient can be made with many different methods and have given highly reliable results [39; 96; 154; 157; 217] for some time [81; 158]. Indeed, nowadays theoretical calculations of activity coefficient are reliable as well, even when made with primitive models like the **MSA** [38; 114; 162], provided the **MSA** is renormalized by the proper choice of parameters [181-185]. (The renormalization is of course the source of problems; but a large number of experimental curves, containing an even larger number of experimental measurements, are fit to considerable accuracy with just a few parameters adjusted in the renormalization procedure.) Equilibrium simulations using **MC** methods reproduce experimental results quite well [10; 50; 51; 166; 185] Indeed, simulations of nonequilibrium molecular dynamics in our lab are now able to reproduce the pair correlation functions of some systems quite well, and thus by implication the thermodynamic properties of those systems [1; 202], although these comparisons have so far only been done in a preliminary way. (Other groups have made comparisons at equilibrium.) In my view, the **SPM** would be even more accurate than the **MSA** if it were normalized with as much care as the **MSA** has been, because the **SPM** is more realistic.

Preliminary Work

Preliminary Work for Specific Aim #1 is found in Background
Preliminary Work for Specific Aim #2 is in Background and (mostly) in the Research Plan

Preliminary Work: Specific Aim #3: Site-directed mutagenesis of *OmpF*

The following section is quite detailed so the experience and expertise of our collaborators can be evaluated. The PI himself is not trained in molecular biology and is fortunate to work with colleagues who are both trained and experienced in these techniques. Preliminary work [129] shows that we can design, make, and measure the properties of mutants of *ompF* .

We work with the gene of porin called ompF so we can modify the protein easily. To facilitate protein purification the leader sequence was removed and a ribosome-binding site and start codon were inserted directly prior to the sequence encoding mature *ompF*, resulting in the plasmid pG*ompF*-mature. This procedure resulted in the protein being expressed in large amounts in inclusion bodies, from which it was purified as described below. The plasmid pG*ompF*-mature was used as template in the QuikChange®XL protocol from STRATAGENE (La Jolla, CA). First, the template was duplicated using reverse complement primers, containing the mutation of interest, resulting in mutated plasmid with staggered nicks. Then, the template was degraded and the mutated plasmid (with staggered nicks) was transformed to E. coli. Sequencing the DNA verified that the resulting mutated genes were only mutated in the intended codon and did not contain additional (silent) mutations. In some cases the ampicillin resistance marker was replaced with a marker for tetracycline. Plasmid

pG*ompF*—encoding the *OmpF* protein after the PhoE leader sequence [155]—was kindly provided by Prof. T. Schirmer, Biozentrum, Basel.

***OmpF* isolation and purification** One liter cell cultures of E. coli Bl21(DE3) or Omp8 [155], containing the desired plasmid, were grown overnight in TY-media supplemented with ampicillin (100 μg/ml) or tetracycline (5 μg/ml). Cell lysis was induced by the addition of lysozyme (0.35 mg/ml) and 1% Triton X-100. After sonication (3x30 s), inclusion bodies were pelleted and washed with 20 mM phosphate buffer, pH=6.5. After centrifugation, the pellet was dissolved in 8 M urea and diluted to a final protein concentration of 0.2 mg/ml in refolding buffer (20 mM sodium phosphate buffer, pH=6.5; 1 mM dodecylmaltoside and 1mM TCEP). After overnight refolding at room temperature, dimer-to-monomer conversion was accomplished by heating to 70 °C for 1 hour. Subsequent degradation of the monomers was induced by the addition of trypsin (trypsin/protein=1/100 w/w). Final purification of trimer protein was achieved by ion exchange chromatography on MonoQ resin (Amersham Biosciences) using an elution buffer containing 200 mM NaPi, pH=8.0, 1% OPOE and 1 mM TCEP. Eluted fractions were analyzed by 12.5% SDS-PAGE (CBB stained) and protein concentration was determined with Lowry (BioRad). Typical total yield was 10-20 mg for WT and 5-6 mg for mutant protein. EAE and LEAE proteins were further purified by extraction out the SDS gel, using elution buffer, giving final protein yields of ≈50 μg.

Partially purified monomeric proteins were derived from inclusion bodies and checked by electron spray mass spectrometry. Theoretically predicted mass and experimentally obtained values were in excellent agreement and differed less than 5 Da.

Solutions. Precise design and understanding of solutions is an essential part of a careful study of selectivity since artifact is easy to generate, hard to avoid, and nearly impossible to remove once present. For that reason some important details are described here. Contamination of Na^+ and K^+ free solutions was avoided by using NMDG instead of NaOH or KOH to titrate the buffer solutions to the desired pH. We therefore compared WT conductance (in symmetrical 1 M KCl, pH 7.4) and selectivity (in 0.1 || 1 M KCl, pH 7.4) in Hepes-buffered solutions, pH-adjusted with either KOH or NMDG. The conductance values of single open trimers was found to be 4.15 ± 0.12 nS (n=11) and 4.20 ± 0.12 nS (n=17) in solutions titrated with KOH and NMDG, respectively. With KOH, the reversal potential was 26.4 ± 1.5 mV (n=15), whereas with NMDG it was 28.2 ± 4.6 mV (n=9). These data indicate that NMDG itself has no significant effect on either conductance or reversal potential.

Activity coefficients were calculated with Geochem-PC 2.0 software [150]. At relatively low ionic strength (<0.5 M), Geochem calculates activity coefficients with the extended Debye-Hückel (or Davies) equation, whereas at higher ionic strength (>0.5 M) a modification of the Helgeson equation is used. Where appropriate, ion activities calculated by Geochem are given in figure legends.

Preliminary Work for Electrophysiology. We measure binding of ions to proteins by measuring current flow through channels. This measurement has special advantages. Biologically, many channels are specialized for binding and thus these binding phenomena are optimized by evolution. Technologically, measurements of current flow through single channels allows high resolution. Binding to **single** protein molecules can be easily studied in channels, and in few other proteins with such resolution. Current flow through channels can be measured by highly developed technology that allows rapid measurement of binding (assayed by the amount of current). The current is easy to measure because **single channel current is a biologically amplified binding assay**: channels evolved to allow macroscopic currents to be controlled by atomic scale binding. Conditions for binding can be changed over a wide range simply by changing the electrical potential across the channel. Potential can be swept over some 200 mV every second with no difficulty, energetically equivalent to changing concentrations some 80× (because 200 mV = 8 kT; 10x concentration is roughly 1 kT of energy).

Finally, concentrations of agonists can be changed as in biochemical experiments with suitable apparatus we have designed and made available commercially [189; 192; 194].

Details of Electrophysiology. To compare theory and experiments, precise measurements of current voltage (IV) curves are essential if selectivity is to be reliably reported. We find that many workers trained in molecular biology are not as aware of the pitfalls of electrophysiology as they might be, so I include here a rather precise description of our procedures taken from our paper reporting the background work for this proposal [129]. Planar Lipid Bilayer (PLB) experiments were performed using a chamber and Delrin cuvet (Warner Instruments Corporation, CT. Models BCH-22A and CD22A). The Cis compartment was connected to the headstage by 3 M KCl/2% agar salt bridges, and the Trans compartment was connected to ground. The PLB was painted across a 250 μm diameter aperture and was composed of phosphatidylethanolamnie (PE) and phosphatidylcholine (PC) in an 8:2 ratio, dissolved in n-decane (10 mg/ml).

Fig. 2

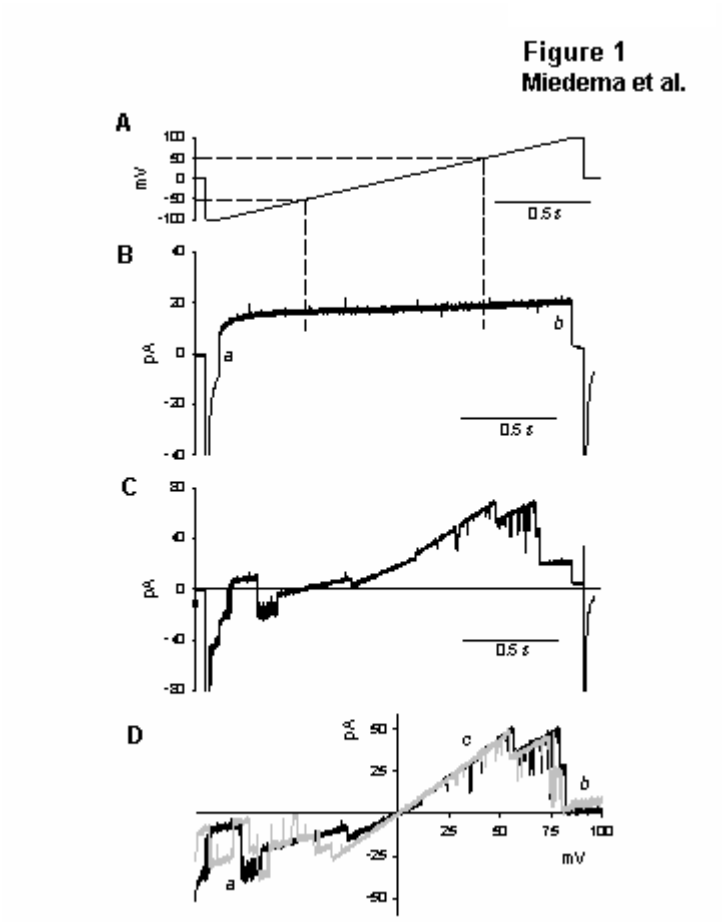


Fig 2. Recording protocol for *ompF* conductance and selectivity measurements. (A) Voltage-ramp protocol. From a holding potential (HP) of 0 mV, the potential (V) was clamped at –100 mV for 0.1 s, after which V was ramped to 100 mV in a little less than 2 s (dV/dt=111 mV/s). After 0.1 s at 100 mV, V was set back to 0 mV. (B) Control recording in the absence of protein with 0.1 M NaCl, pH 7.4 at both sides of the membrane. The instantaneous current jumps (ΔI) at the onset and offset of the voltage ramp, indicated by a and b, represent charging and de-charging of the membrane capacitance, C_m (≈150 pF, according to $\Delta I=C_m dV/dt$). That part of the control trace between -50 and 50 mV (dashed lines) was fit by linear regression and subtracted from traces as shown in C with *ompF* in the bilayer. (C) Current trace with *ompF* (EAE mutant) reconstituted in the bilayer and before correction. (D) Corrected current-voltage or IV-plot derived from the data in C. A second IV-plot (in grey) is shown to highlight the existence of subconductance states (indicated by a), the residual conductance after *ompF* 'closure' (b) and the current level with all three monomers fully open (c).

The pulse protocol used (Fig. 2A) started from a holding potential (HP) of 0 mV. After the potential (V) was jumped to -100 mV, V was held at -100 mV for 0.1 s, after which V was ramped to 100 mV in a little less than 2 s. Finally, after 0.1 s at 100 mV, V was jumped back to 0 mV. Fig. 2B shows the current recorded from the PBL in the absence of protein, with 0.1 M NaCl, pH 7.4 on both sides of the membrane. The instantaneous current jumps (ΔI) at the beginning and end of the voltage ramp—indicated by a and b in Fig. 2B, respectively—represent the rapid charging and de-charging of the membrane capacitance C_m. That part of the trace in between -50 and 50 mV was fitted by linear regression (dashed lines) and subtracted from 'raw' traces measured with *OmpF* in the bilayer. Fig. 2C shows raw data; Fig. 2D shows corrected data. The control recording in Fig. 2B is important for several reasons. Firstly, it allows a correction for any seal and/or

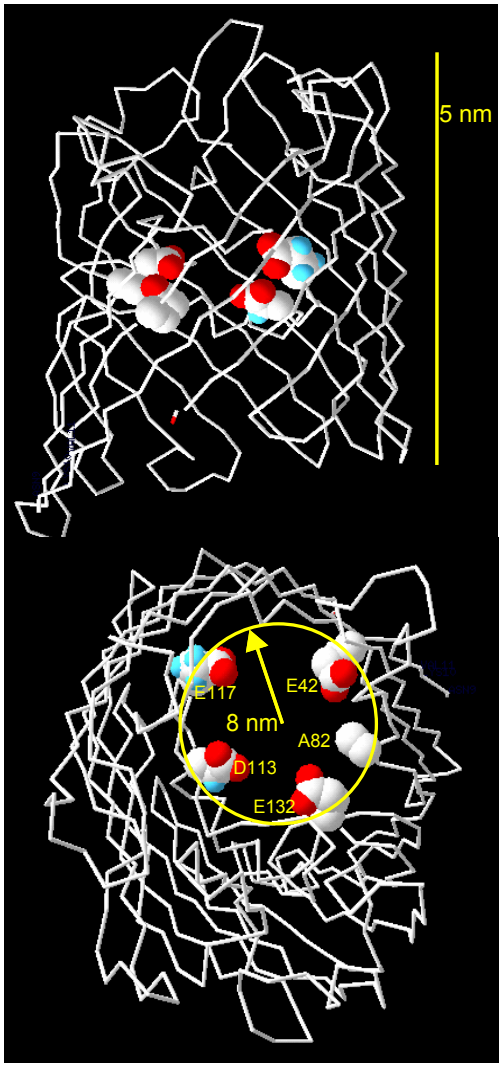
membrane leak conductance (R_m) as well as for the capacitive transients mentioned above. Secondly, it allows the assignment of the zero current level with the trimer completely shut, not a triviality because of the frequently observed residual conductance that remains after apparent closure of all three monomers (compare sections b of the two IV's in D). Finally, the followed procedure of current subtraction effectively corrects for current offsets.

Correct handling of offsets is crucial for these measurements. The value of shunt resistance R_m depends on ionic conditions, notably Ca^{++} concentration. It is essential therefore to obtain control recordings under exactly the same ionic conditions as the experimental recordings. The practical implication is that it is usually necessary to perform separate sets of experiments for each ionic condition, instead of changing solutions during an experiment.

Potential differences (V) are defined as $V=V_{cis}-V_{trans}$. A positive (outward) current is defined as an efflux of cations from *cis* to *trans*. In experiments with pre-defined ionic gradient, measured reversal potentials (E_{rev}) were corrected for measured liquid junction potentials (LJP, mentioned in the figure legends where applicable). In addition, experiments were performed with PLBs painted under symmetrical ionic conditions and the gradient applied after membrane formation. Results rendered from both protocols were in excellent agreement.

Conductance *g* means the slope conductance of fully opened trimer protein, with equal concentrations of ions on both sides, at 0 mV (measured over a 50 mV interval ranging from -25 to 25 mV), i.e. at E_{rev}. Conductance was derived from the trimeric current level (c in Fig. 2D) because it can be estimated precisely even in the presence of multiple sub-conductance states (see at a in Fig. 2D).

Fig 3. *ompF* Selectivity Filter



<u>Preliminary work: calculations of current in *ompF* and mutants</u>. The calculations have that will be done for the proposed project are shown below in some detail. Summaries of our previous work with the crowded charge model are in the background and significance section but details are not given because the work has been published [54; 55; 59; 143; 147].

We consider six formal charged residues present in the constriction zone of WT *OmpF*: K16, R42, R82, R132, E113 and D117. It was assumed that the arginines (pK~12) and lysines (pK~10.4) all have charge +1e and the glutamates and aspartates (pK's~4.4) all have a charge of –1e, i.e., these residues are assumed to be either fully protonated or ionized [206]. Apart from these six charged residues in the constriction zone of *OmpF* (with a net charge of +2e), negatively charged amino acids positioned in the entrance of the channel line the pore wall and contribute to the overall net negative electrostatic potential, thereby causing WT *OmpF* to be slightly cation selectivity [101; 102; 152]. It is for this reason that MD calculations consistently included a surplus of monovalent cations [101; 102; 187]. In order to ensure that WT in our calculations behaves as a (slightly) cation-selective channel we assumed the existence of three additional negative fixed charges, resulting in a net charge of -1e. These three residues are supposed always present—in WT and in mutant protein—smeared out uniformly over the wall of the selectivity filter and therefore not modeled explicitly. The

net charge of WT and the recombinant proteins used in this study are listed in Table 1 of [129]. Experiments at pH 9 and 3 confirmed the ionization state of the residues involved (see also [139]). Whereas recordings at pH 9 were essentially identical to those at pH 7.4, differences between WT and mutant protein vanished at pH 3, presumably because the newly introduced glutamates were protonated ("neutralized") at such acidic pH.

Conductances, all in symmetrical solutions, were computed using a combination of density functional theory (**DFT**) and Poisson-Nernst-Planck (**PNP**) theory. Local excess chemical potentials of particles computed in the **DFT** are incorporated into the chemical potentials of the ions and water; these chemical potentials were included in the **PNP** description of ion flow. The approach followed has been described in detail [71; 72]. The crowded charge term in the model depends steeply on the volume of the pore, i.e., the amount of crowding, as one would expect intuitively. Therefore, apart from the fixed charge, another critical input parameter is the volume of the selectivity filter. We do not have the crystal structures of these mutant *OmpF* proteins at hand, and are just now (Sept 2004) discussing determining those structures in collaboration with my colleague and friend Tilman Schirmer in Basel. For now, we estimate the filter volume. Fig. 3 shows a top view and a side view of a model of the EAE mutant with E42, A82, E132, D113 and E117 indicated. This model was created by simply substituting the side chains of the implicated residues without further refinement of any kind. Distances between the oxygens of E42 and D113 and between the oxygens of E117 and E132 are approximately 1.6 nm. Based on these numbers, we hypothesized the pore to be formed by a cylinder with a radius of 0.8 nm and a length of 1 nm, enclosing a volume of approximately 2 nm^3. This central part is flanked by two symmetrical, conical atria (1 nm long, cone angle 45 degrees) and embedded in a membrane 3 nm in thickness. The carboxylate groups of E113 and D117 and the guanidinium groups of K16, R42, R82 and R132 are represented as charged spheres 0.45 nm in diameter. These formal charged residues are modeled as ions that are confined to the cylindrical part of the pore but are otherwise free to move within the cylinder. The mobile, permeating ion species are represented as charged hard spheres (using the crystal diameters given in Table 1 of [140]), and water as uncharged spheres (diameter 0.28 nm) in a uniform dielectric with a relative permittivity of 80. Diffusion coefficients are assigned bulk values, except in the pore; here, D_{Na}, D_{Ca}, and D_{Cl} (in units of $10^{-11}m^2s^{-1}$) were 6, 0.6, and 6 (WT), or 1.5, 0.2, 1.5 (EAE and LEAE). The ratio of D_{Na} and D_{Ca} in the pore was chosen to be ~10 ($=D_{Na}/D_{Ca}=2g_{Na}/g_{Ca}$, see [70]) and inferred from a measured g_{Na} of ~2.5 nS (in 1 M NaCl, see Table 2 of [129]) and a g_{Ca} of ~0.5 nS (in 0.1 M $CaCl_2$ [71]). Conductances were computed from the current at 10 mV applied potential and the **DFT**/**PNP** equations were solved as described in [71]

Theoretical calculations were made of conductance under symmetrical ionic conditions. Although the ratio D_{Na}/D_{Ca} was estimated from experimentally obtained data, their absolute values remain unknown. The theory computes partitioning but not diffusion coefficients; the latter are input parameters needed to calculate flux. The shape of Anomalous Mole Fraction [94] curves (see Fig 4 & 5) at end of proposal) is determined by Ca^{++} dependence of Na^+ current which, in turn, depends on the partitioning of both ion species in the pore. Diffusion constants, on the other hand, only scale conductance without affecting overall shape. Therefore flux calculations are far less prone to the poorly known estimates of diffusion constants inside the channel than calculations of E_{rev}. Diffusion coefficients may have qualitative effects on E_{rev}, i.e. they can change the polarity of E_{rev} and for that reason E_{rev} calculations are not shown here and have not been reported.

Research Design and Methods

Our methods are designed to combine theory, simulation, molecular design, and channel measurement to allow design of Ca^{++} selective channels. In that way we build something new and useful scientifically and also evaluate the working hypothesis that crowded charge effects are the dominant determinant of selectivity.

Research Design of Specific Aim #1: using existing models of Selectivity. See Background Section.

Research Design of Specific Aim #2: Improved models of Selectivity are described in the next pages. Existing models of selectivity are described in the Background Section.

We propose to improve our **MSA**, **SPM,** and **DFT** models of ion selectivity and permeation (1) by using better models of water, including (for example) cohesive forces between particles and (2) by including the important dielectric boundary force **DBF** that arises from polarization charge at interfaces between different materials.

Research Design of Specific Aim #2.1: Better models of water. We propose to improve the Solvent Primitive Model by replacing the hard sphere representation of water with more realistic representations that allow cohesive forces between all particles, e.g., between ions, water molecules, and parts of proteins and lipid. The cohesive forces can be modeled as Lennard-Jones, square-well, or Yukawa potentials, all of which give qualitatively similar results and quantitatively nearly identical results if they are calibrated to have identical overall cohesive and repulsive interatomic forces. Analytic results for Yukawa potentials already exist in the **MSA** and other models [18; 20; 159-161]. If necessary, we will renormalize each model by choosing parameters to fit experimental data, following the Simonin approach we have used before [181-183; 185]. Other renormalization schemes may have advantages and we will not hesitate to use them, if need be [10].

We now present details of our most analytical approach using cohesive forces in a reasonably self-contained form. In our calculations we will assume that all particles are hard spheres with cohesive interactions and the usual electrostatic (Coulomb) outside the hard core. (Our publications do not include cohesive interactions, cf. [21; 23; 70; 71; 73; 140; 146]):

$$u_{ij}(r)=\begin{Bmatrix}\infty & \text{if } r\le\sigma_{ij}\\ u_{ij}^{\text{cohesive}}(r)+u_{ij}^{\text{ES}}(r) & \text{if } r>\sigma_{ij}\end{Bmatrix}. \tag{1}$$

For the Lennard-Jones particles, the cohesive interaction potential then is

$$u_{ij}^{\text{cohesive}}(r)=4a_{ij}\left[\left(\frac{b_{ij}}{r}\right)^{12}-\left(\frac{b_{ij}}{r}\right)^{6}\right], \tag{2}$$

while for the Yukawa particles, the cohesive interaction potential is

$$u_{ij}^{\text{cohesive}}(r)=\alpha_{ij}\frac{\exp\left(-\beta_{ij}r\right)}{r}. \tag{3}$$

The parameters a_{ij}, b_{ij}, α_{ij}, and β_{ij} will be determined to yield accurate (mean) bath activities in bulk solutions. These activities have been accurately known from measurements by several methods for a century [39; 81; 96; 154; 157; 158; 217].

In *homogeneous* (i.e., bulk) solutions, the chemical potential of ion species has an ideal gas and an excess component, beyond the ideal gas:

$$\mu_i=\mu_i^{\text{id}}+\mu_i^{\text{ex}}=\ln\left(\frac{\rho_i}{\rho^*}\right)+\mu_i^{\text{ex}} \tag{4}$$

ρ_i is the concentration of species i and ρ^* is a concentration scale (e.g., 1 M). The formalism to determine bulk excess chemical potential is based on the well-known Ornstein-Zernike equation [15; 48; 66; 166]

$$g_{ij}(r)-1=c_{ij}^{(2)}\left[\left\{\rho_i^{\text{bulk}}\right\};r\right]+\sum_k\rho_k^{\text{bulk}}\int c_{ik}^{(2)}\left[\left\{\rho_i^{\text{bulk}}\right\};\left|\mathbf{r}-\mathbf{r}'\right|\right]\left(g_{ik}\left(\left|\mathbf{r}'\right|\right)-1\right)d\mathbf{r}' \tag{5}$$

That must be solved with another equation (the "closure"). The most general closure [159; 160] known to us is

$$g_{ij}(r)=\exp\left(-u_{ij}(r)-B_{ij}(r)+\sum_k\rho_k^{\text{bulk}}\int c_{ik}^{(2)}\left[\left\{\rho_i^{\text{bulk}}\right\};\left|\mathbf{r}-\mathbf{r}'\right|\right]\left(g_{ik}\left(\left|\mathbf{r}'\right|\right)-1\right)d\mathbf{r}'\right) \tag{6}$$

where $B_{ij}(r)$ is the "bridge function." Various approximations are available to the bridge function (e.g., the hypernetted chain and the formulation by Rosenfeld just cited). Another approximation for the closure is the Mean Spherical Approximation (**MSA**) that assumes that the second-order **DCF** is the asymptotic limit (i.e., determined after subtracting the interaction potential) at all locations outside the contact distance:

$$c_{ij}^{(2)}\left[\left\{\rho_k^{\text{bulk}}\right\};\left|\mathbf{r}-\mathbf{r}'\right|\right]=-\frac{1}{kT}u_{ij}\left(\left|\mathbf{r}-\mathbf{r}'\right|\right)\ \text{for}\ \left|\mathbf{r}-\mathbf{r}'\right|>\sigma_{ij}\quad\text{where}\ \sigma_{ij}=\frac{\sigma_i+\sigma_j}{2}. \tag{7}$$

The **MSA** has the advantage that it usually gives analytic formulas (at the expense of some accuracy, of course). Several results are known for charged Yukawa fluids in the **MSA** [18; 84; 86; 87; 106; 159-161].

For an *inhomogeneous* fluid (i.e., where the particle densities are location-dependent), the chemical potential in Eq. (4) is now location-dependent because the concentrations are now location-dependent. **DFT** computes the excess chemical potential at every location by minimizing the grand potential of the system:

$$\frac{\delta F}{\delta\rho_i(\mathbf{x})}=0. \tag{8}$$

The grand potential F has an ideal gas and an "excess" (beyond an ideal gas) component:

$$F\left[\left\{\rho_k(\mathbf{y})\right\}\right]=F_{\text{id}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]+F_{\text{ex}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]. \tag{9}$$

With all particles having a hard core, we split the grand potential into the hard sphere **HS** and long range (beyond the hard core) components:

$$F_{\text{ex}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]=F_{\text{HS}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]+F_{\text{LR}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]. \tag{10}$$

Excellent approximations of the **HS** component are well-known [159; 160; 163-165]. The long range **LR** component is not as well established, but the **DFT** framework we have developed for the electrostatic component [71; 72] allows for straight-forward generalization to arbitrary long-range interaction potentials. This **LR** functional is based on a perturbation about an inhomogeneous reference fluid:

$$\begin{aligned}F_{\text{LR}}\left[\left\{\rho_k(\mathbf{y})\right\}\right]\approx F_{\text{LR}}\left[\left\{\rho_k^{\text{ref}}(\mathbf{y})\right\}\right]-kT\sum_i\int c_i^{(1),LR}\left[\left\{\rho_k^{\text{ref}}(\mathbf{y})\right\};\mathbf{x}\right]\Delta\rho_i(\mathbf{x})\,d\mathbf{x}\\ -\frac{kT}{2}\sum_{i,j}\iint c_{ij}^{(2),LR}\left[\left\{\rho_k^{\text{ref}}(\mathbf{y})\right\};\mathbf{x},\mathbf{x}'\right]\Delta\rho_i(\mathbf{x})\Delta\rho_j(\mathbf{x}')\,d\mathbf{x}\,d\mathbf{x}'\end{aligned} \tag{11}$$

where

$$\Delta\rho_i(\mathbf{x})=\rho_i(\mathbf{x})-\rho_i^{\text{ref}}(\mathbf{x}) \tag{12}$$

and first and second order direct correlation functions (**DCF**s) are functional derivatives of the grand potential:

$$\frac{\delta F_{\text{LR}}}{\delta\rho_i(\mathbf{x})}=-kTc_i^{(1),LR}\left[\left\{\rho_k(\mathbf{y})\right\};\mathbf{x}\right] \tag{13}$$

$$\frac{\delta^2 F_{\text{LR}}}{\delta\rho_i(\mathbf{x})\delta\rho_j(\mathbf{x}')}=-kTc_{ij}^{(2),LR}\left[\left\{\rho_k(\mathbf{y})\right\};\mathbf{x},\mathbf{x}'\right] \tag{14}$$

Taking the functional derivative of the **LR** functional, the first-order **DCF** of the fluid is determined:

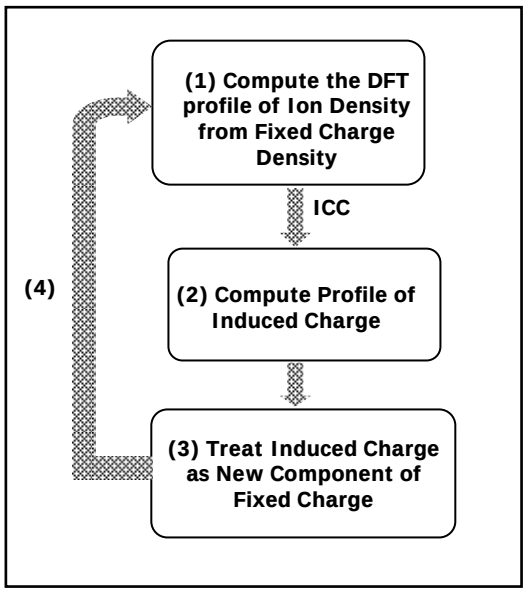
$$c_i^{(1),LR}\left[\left\{\rho_k(\mathbf{y})\right\};\mathbf{x}\right]\approx c_i^{(1),LR}\left[\left\{\rho_k^{\text{ref}}(\mathbf{y})\right\};\mathbf{x}\right]+\sum_j\int c_{ij}^{(2),LR}\left[\left\{\rho_k^{\text{ref}}(\mathbf{y})\right\};\mathbf{x},\mathbf{x}'\right]\Delta\rho_j(\mathbf{x}')\,d\mathbf{x}' \tag{15}$$

The first-order DCF is the excess chemical potential component of the inhomogeneous fluid.

To determine the first-order **DCF**, we need a reference fluid and the first- and second-order **DCFs** for this reference fluid. This is done by averaging the current fluid densities at every location and applying bulk formulas for the two **DCFs** at every location.

Once $c_{ij}^{(2)}\left[\left\{\rho_i^{\text{bulk}}\right\};r\right]$ for the bulk has been computed, $c_i^{(1)}\left[\left\{\rho_k^{\text{bulk}}\right\};r\right]$ is computed by functional integration. With bulk functions computed, the **LR** functional is completely defined and **PNP/DFT** theory can be used to compute flux of ions through channels as described in papers [23; 71; 72; 146] and in the Background Section.

Research Design of Specific Aim #2.2 Dielectric interfaces.



Dielectrics are easy to include in bulk models because bulk solutions are homogeneous and their polarization can be approximated by one number, the dielectric constant. **In**homogeneous systems—like ions in channels—are more difficult to handle because significant charge and force arise at dielectric interfaces (solution|lipid, solution|pore, pore|protein, etc.) The **DFT** is designed to deal with inhomogeneous systems but (as far as we know) dielectric boundaries have not been included in **DFT** to date. Including different dielectrics will be a significant contribution to the theory of inhomogeneous of fluids, if we can do it.

We have previously computed the charge induced on dielectric interfaces with our Induced Charge Computation (**ICC)** method. We can extend this treatment to include dielectric boundaries in the mean field by an iterative method:

#1 compute the **DFT** ion density profile without a dielectric interface;

#2 compute the induced charge profile (using the **ICC**) from the ion density profile computed in #1;

#3 compute the **DFT** ion density profile with an additional component of fixed charge, namely the induced charge profile just computed in #2;

#4 iterate, until converged. (Iterations like this have converged quickly in our previous work [33; 71; 144]).

This mean field approach would be improved [32; 44; 45; 53; 75; 112; 136-138; 176; 177] by a discrete charge treatment of the dielectric boundary force **DBF** on charged points or spheres. The **DBF** on a charge of strength *q* at location $\mathbf{r}_1$, where the dielectric coefficient is ε_1, is [138; 176]

$$\mathbf{F}(\mathbf{r}_1) = -q\nabla_r \left(\Phi(\mathbf{r}) - \overbrace{\frac{q}{4\pi\varepsilon_1\varepsilon_0|\mathbf{r}-\mathbf{r}_1|}}^{\text{Point Term}} \right)_{\mathbf{r}=\mathbf{r}_1} \tag{16}$$

where the '***Point Term'*** is present when the charge *q* is a point at $\mathbf{r}_1$ to remove a singularity otherwise present. If the charge were on a sphere of any radius [138; 176; 177] at $\mathbf{r}_1$, this term would not be present. The potential $\Phi(\mathbf{r})$ is given by the following Poisson equation, with potential vanishing at infinity, and with the charge distribution $\rho(\mathbf{r})$ in a region surrounded by the surface $\partial\Omega$ where the dielectric coefficient is $\varepsilon(\mathbf{r})$

$$\nabla\cdot\left[\varepsilon(\mathbf{r})\nabla\Phi(\mathbf{r})\right] = -\frac{1}{\varepsilon_0}\rho(\mathbf{r}) \tag{17}$$

and

$$\left[\varepsilon(\mathbf{r})\nabla\Phi(\mathbf{r})\cdot\mathbf{n}\right]_{\partial\Omega} = 0; \quad \Phi(\mathbf{r}) = 0 \text{ as } |\mathbf{r}|\to\infty \tag{18}$$

We propose to make a discrete analysis of the dielectric boundary effect by including the appropriate dielectric energy in the functional and variational principles that define the **DFT** and then deriving the appropriate solution of that new variational problem. We are familiar with this process of implementing a variational principle [4; 23; 71] and thus treat new variational principles without fear but with appropriate respect because it will involve definition of the appropriate free energy including dielectric boundary energy, construction of a new variational principle, computation of the functional derivatives, solution of the resulting Euler-Lagrange equations, and consideration of a good numerical method to approximate that solution.

Possibilities….what if the structure Is discovered?

The models shown above are based on current knowledge of the structure of Ca^{++} channels which is quite limited: a great deal is known about primary structure (i.e., sequence of amino acids) and a great deal has been inferred about the active site and the amino acids composing it, [5-7; 40-43; 61; 67; 91-93; 113; 115; 144; 153; 156; 201; 211; 212; 214; 215] but detailed structure is essentially unknown. Structural biologists are working hard to determine the structure of Ca^{++} channels [211; 212] (both the L-type and the ryanodine receptor according to corridor conversations, and probably many other Ca^{++} channels unknown to us). If a structure is determined, we obviously would include that information in our models as quickly as we could.

How would we include structural information that may arise from x-ray crystallography or **NMR** in the next years? One possibility is to extend the **DFT** to three dimensions and such is being done by our colleague and friend who introduced us to the **DFT** and whom we introduced to ion channels some years ago, Laura Frink. [68] Dr. Frink works at Sandia National Laboratory with the enormous computer resources (literally, tens of clusters of thousands of processors each) necessary to do **DFT** in three dimensions, but even so, worked out applications to specific structures and biological problems have not yet materialized. We watch this work with admiration and hope, and have done what we could to help Dr. Frink with her preliminary work on gramicidin. We cannot count on doing with our limited resources what Dr. Frink has not yet been able to complete with all the resources available at Sandia.

So, if a structure is published, we will turn to Monte Carlo simulations using the techniques developed by our collaborators Doug Henderson and Dezso Boda [21-26; 197]. The detailed structure would allow us to pinpoint where the selectivity filter is and calculate calcium binding (i.e., the number density of Ca^{++} ions) in the presence of monovalent cations (for example, Li^+, Na^+, K^+, and Cs^+). Resources would be reallocated to make these calculations possible; this proposal as it stands uses **MC** only for calibration of our various more analytical models. If a structure becomes available, **MC** would assume a much more prominent place in our work, so we could exploit the structural data. Fortunately, I am in continual contact with Doug and Dezso (nearly daily: I am editing the Festschrift for Doug's 70^{th} birthday and we share many personal interests and friends) and have been developing **MC** for our own use, in collaboration with Wolfgang Nonner and his graduate student Alex Peyser: efficient implementation of **MC** code involving dielectric boundary force is part of Alex's thesis and is being done in collaboration with Co-PI Dirk Gillespie.

Monte Carlo is the simulation method of choice for this problem particularly because it is calibrated and known to reproduce experimental results in detail (e.g., [15; 16; 25; 197]). It is also helpful that we are experienced with **MC** and that **MC** is fast and feasible in three dimensions after its optimization by Dezso Boda Dirk Gillespie, Wolfgang Nonner, and Alex Peyser even after the **DBF** [23] was included in the simulations.

As of now, direct simulation using traditional molecular dynamics (with periodic boundary conditions) or self-consistent molecular dynamics [202] or even self-consistent Langevin dynamics [1] is too slow to allow computation of physical components of selectivity (i.e., free energy per mole). ["Self-consistent" means Poisson is solved without periodic boundary conditions every time the distribution of charge changes, see [170].] Indeed, any kind of molecular dynamics known to us is too slow to allow computation of many current

voltage curves, reversal potentials, and thus selectivity in a range of conditions, although my collaborators Marco Saraniti and Umberto Ravaioli and others [40; 41; 110; 111] are working on this. We hope to make sufficient progress to propose a parallel project to the **NIH** using Langevin methods, and perhaps molecular dynamics, with atomic resolution of structure to verify and extend the lower resolution models presented in this application and the equilibrium results of **MC** simulations. We may also use the Lennard-Jones model implemented numerically in one of the forms we used in earlier papers [1; 202] and combine it one way or another with the analytical approach just described. (This numerical treatment is not part of the work described in the Specific Aims here, but it might be done later on, if for example a structure becomes known. Our present plan is to write another proposal asking for separate support for this ambitious project. We describe those plans here in brief to show our understanding of this approach.) If we do this, we must take care to represent the long tail of the distribution (i.e., large time values) accurately: we have found, following many others, that it is essential to have the integral of the tail correct, if we are to reproduce pair correlation functions determined from high resolution models (e.g., hypernetted chain **HNC** or **MC** simulations) (as we did in the work just cited).

Cut-offs of Lennard Jones potentials must be chosen carefully, if they are used at all, and tapers in the interatomic potentials must be designed to have the right area. Problems in using Lennard Jones potentials to compute pair correlation functions are closely related to problems in computation of Fourier transforms in general. Briefly, the problems are serious because cut off errors are produced when any integral over an infinite domain is truncated (in finite Fourier transforms these are called 'Gibbs Phenomena') and these interact in a nasty way with discretization effects [30; 31] unless the density of discretization is enormous (say $>10^6$ samples per unit of independent variable). These problems had to be faced by the PI in an earlier life, when he used Fourier transforms (evaluated in hardware) to measure the impedance and equivalent circuits of skeletal and heart muscle and lens of the eye) [60; 116; 124; 125]. Measurements of impedance of real analog circuits made of real resistors and capacitors clearly showed how errors of truncation and discretization could bias results if they were not treated carefully. In recent work on ion channels, these problems arose again [1; 202], in somewhat different form, and were solved again, but further subtleties may arise in the implementation of Lennard Jones. We are glad we can turn to our friends and collaborators Doug Henderson (**BYU**), Andrij Trokhymchuk, and Dezso Boda, who have worked in this area for years, for advice and help.

The Yukawa representation of cohesive forces has not been used as much as Lennard-Jones, and we expect unforeseen difficulties in its implementation. Fortunately, Lesser Blum (who is one of the leaders in Yukawa potentials) has worked with us in the past, teaching us the **MSA** in fact, visiting my Department for a month on occasion. His help will be most useful as we use his Yukawa representations in new ways.

Research Design of Specific Aim #3: Site Directed Mutagenesis. *(Figures and Tables are at end of this Section)* Using conventional site directed mutagenesis (details in Background Section), we will construct and test mutant proteins with the high densities of carboxyl groups and the small pore volumes needed to produce physiological Ca^{++} selectivity, according to the models and simulations of Specific Aims #1 and #2. Our preliminary work presented above generated and characterized a mutant EAE of bacterial porin *ompF* in which three positively charged arginines (R) in the selectivity filter (i.e., active site) were replaced by two glutamates (E) and one neutral alanine (A). It is clear that our methods allow the expression, isolation, and measurement of mutant porins with very different properties in their active site, particularly with very different charge densities in the active site. Fortunately, the active site we are manipulating is in a robust protein from an intestinal bacteria, which survives in the stomach (in dilute HCl, nearly), in the intestine (in nearly neutral acidity), and in the outside world, even when dried to a powder. Indeed, we chose *ompF* for our work because it is such a strong protein, resisting denaturation even at high temperatures (e.g., wild type is stable at some 60°C). Porin is so robust because it interacts with the outer world (mostly) through its outer wall which is a β sheet (one of

the strongest protein structural motifs) and (we suppose) because its active site is so far from its outer wall. For whatever reason, it is a fact that *ompF* is a quite easy protein to handle [46; 104; 118; 167; 168; 172; 189], as are its mutants, and we (and many others) have had relatively little difficulty expressing mutants, incorporating them into bilayers, particularly compared to eukaryotic membrane proteins.

The EAE mutant has been easy to handle even though it has a very different electrostatic profile from the wild type *ompF*. Three positively charged arginines (R) in the selectivity filter (i.e., active site) of *ompF* are replaced by two glutamates (E) and one neutral alanine (A) in the active site EAE. The fixed charge of these three residues was changed from +3 to −2, a net change of charge of −5*e* (assuming full ionization of acidic and basic residues, as seems likely from the careful analysis of [206]). The resulting negatively charged selectivity filter of the EAE mutant produces a high cation over anion selectivity, as would be expected. Thus, the mutant has high selectivity for Ca^{++} compared to anions, unlike wild type porin, which has almost none. Nonetheless, we have not made a good Ca^{++} channel yet.

The mutant EAE channel is not highly selective for Ca^{++} over Na^{+} as a good Ca^{++} channel must be if it is to pass mostly Ca^{++} current, in physiological solutions. The different properties of the EAE mutant compared to the L-type Ca^{++} channel is seen clearly in the classical anomalous mole fraction plot shown in Fig. 4A (see discussion in [94]. The channel is not 'blocked' by Ca^{++} as is the cardiac channel. (Note that anomalous mole fraction effects can be produced in a number of ways in our selectivity models [70; 142] and our model of the L-type Ca^{++} channel does of course show anomalous mole fraction effects [21; 33; 140-144; 146]).

It seems clear why the mutant EAE channel differs from the L-type Ca channel. The EAE pore is too wide, the volume of the pore (i.e., the selectivity filter) is too large and, consequently, the charge density in the pore is too low (Fig. 4B). It seems intuitively obvious that crowded charge effects require small pore volumes and this is just what theory shows (e.g., Fig. 8 of [129]; see Background Section of this proposal). Table 1 compares properties of the L-type Ca-channel (L-Ca) with those of the EAE mutant.

Our goal is to create mutations that make EAE more selective by reducing its volume (see Fig. 5). According to our theory of crowded charge, crowding becomes a key determinant in ion selectivity when the packing fraction (i.e., the fraction of pore volume occupied by ions, including the eight carboxyl oxygens of the EEEE-locus) is 0.25 or more [140]. The packing fraction in the L-type Ca-channel (L-Ca) falls in this range. The estimated volume of the EAE mutant is more than 5× larger than that of L-type Ca channels (~2 nm^3 vs. <0.4 nm^3; see Table 1). (We estimate dimensions of the mutant as 1.6 nm × 1 nm, volume 2 nm^3 . These are crude estimates. The length dimension 1 nm is based on the *z*-coordinates of the Cα atoms of the five key residues in the known crystal structure of wild type porin *ompF*. The pore length is overestimated, we hope only slightly, because it neglects the pointing of the carboxyl oxygens and guanidinium groups towards the center of the pore). To make a proper Ca^{++} channel, we need a packing fraction in a new mutant to be ~0.25 which means reducing the volume to <0.5 nm^3. The choice of substitution is determined by the residual volume of the relevant amino acids (see Table 2). Obviously, the most effective substitution would replace the smallest alanine by the most bulky one, tryptophan, resulting in a volume reduction of 0.139 nm^3. The amino acids suitable for this substitution must be part of the constriction zone and point into the pore lumen. Few amino acids satisfy this criteria and that is probably why the volume reduction produced by our amino acid substitutions have not yet been sufficient to substantially increase the packing fraction. For instance, the triple mutation A82W, A123W, V337W reduces the volume by no more than 0.37 nm^3, resulting in a final filter volume of 1.63 nm^3. This analysis is discouraging but must not foreclose further experimental work. If we are unable to reduce volume sufficiently, we will turn to other proteins, e.g., maltoporin or omp32, or hemolysin filled with molecular adapters (see Section at end of Research Design: **What if we fail with ompF**?)

We will investigate further mutations by structural analysis using the wonderful visualization software available for this purpose. (We like VMD and Swiss PDB). Specifically, (1) we will look for substitutions that might force bending of the structure (2) we will study the homology with maltoporin looking for ways to mimic the narrow pore of this closely related protein (3) we will keep asking colleagues and friends and searching the literature for mutants that are highly selective (for anything!) or have other indications of narrower pores.

While this empirical search is not as logical as we wish, because the protein folding problem is unsolved, it should not be dismissed. A substantial fraction of successful mutants have been designed this way, and a large fraction of our knowledge of structures has been based on model building with such qualitative approaches (although I certainly wish for a more rational basis for our planning).

We now consider an alternative strategy to make more selective channels. We consider increasing packing density and reducing pore volume by making chemical modifications of the active site of porin *ompF*.

Research Design of Specific Aim #4: Chemical modification. The obvious way to chemically modify *ompF* is to take advantage of the sulfhydryl **–SH** group in the naturally occurring amino acid cysteine. This strategy has been widely used for decades and is often successful, but often only after a substantial amount of experimental tinkering. (The analogy I use to describe this process to my mathematical colleagues is that of baking a soufflé in a well insulated convection oven at a decent altitude, say 1,500 meters. Everything needs to be changed in the recipe that Julia Child perfected in Boston, near sea level in a conventional uninsulated oven. But nothing should be changed too much!). *OmpF* has no cysteine residues so the first step is to place cysteines in the pore in the constriction zone that makes the selectivity filter and active site. We propose (and in fact have already started) replacement of the three arginines of the constriction zone, R42, R82 and R132, with cysteines, making a CCC-mutant. This approach has several advantages:

First, we do not expect any particular problems in expressing or isolating or reconstituting the mutant CCC protein because we have previously expressed, isolated, and reconstituted the AAA mutant (with mutations R42A, R82A, R132A) without complication. Second, the R82C mutant is already available and can be expressed in large amounts in inclusion bodies in our expression system (see details in the Background Section) because the pG*ompF* plasmid lacks the leader sequence. Indeed, we have already made, expressed, isolated, and chemically modified one of these introduced cysteines (C82), see Fig. 4 & 7.

Once the cysteines are in the mutant protein, it is necessary to chemically modify them. This has not caused problems in the preliminary work just cited. Two chemical modifications will be used. One modification will use the relatively slowly reacting maleimide-based compounds (e.g. MIANS); others will use the more reactive thiosulfonates (MTS-based) (Fig. 6). We will start with the maleimide-based labels because they are bulky with an estimated occupied volume of ~0.5 nm^3. (They are about twice as large as tryptophan, see Table 2). Ideally, the labeling of all three cysteines will decrease volume by ~1.5 nm^3, leaving the final filter space as small as 0.5 nm^3, what we think we need. If the volume reduction proves insufficient, one or two tryptophans (A123W and possible V337W) will be introduced to this CCC-mutant.

Any decrease in volume that increases selectivity will suggest that crowding is involved in selectivity, even if we are disappointed and do not succeed in creating a channel as selectivity as the natural cardiac L-type Ca channel. Of course, other interpretations of the effects of volume decrease are possible. The strongest evidence for the crowded charge model comes from the combination of specific theory and detailed experiments, based on the known physical chemistry of ionic solutions.

The MTS compounds also have advantages because of the large variety of thiosulfonates commercially available (+/- charged; short/long, see Fig. 6). Moreover, MTSET, MTSEA and MTSES are known to permeate through pores as narrow as 0.6 nm [107; 188], i.e., almost three times as narrow as the 1.6 nm EAE pore.

A number of complications can arise in these experiments. We have to be careful to label all three of the subunits of porin because this protein is a 3-pack of channels: its 'molecule' has three pores in parallel, and incomplete labeling will make experiments hard to perform. Forcing labeling to completion simply means using somewhat more vigorous reaction conditions, and longer incubations, and we anticipate minor struggles with this, but no worse than that. We also have to be concerned that the target site might prove inaccessible to the label. If this turns out to be a problem indeed, we will consider performing the labeling prior to protein folding. We do not anticipate the need to react before folding, however, because a glance at the structure of *ompF* shows that it has antechambers of large diameter (>2 nm) in very close (~0.2 nm) proximity to the constriction site. These antechambers are known to quickly exchange with bulk solution and allow reagents easy access to the cysteines we wish to react.

What if we do not succeed with ompF? It is sadly possible that we will not succeed in reducing the volume of ompF sufficiently with the methods outlined. If after a number of years following the plan outlined, we have not achieved the selectivity of natural L-type Ca^{++} channels, we will switch to a different protein and modify it.

We are fully aware that each new protein requires mastery of the special properties of its plasmid, of expression of the protein, harvesting, handling, reconstitution, manipulation of gating and recording of current voltage relations, and we do not take such tasks lightly. Nonetheless, we have faced these issues many times in the past with many different proteins and mutants and usually have solved them. Henk Miedema has much experience with a wide range of channel types [65; 127; 128; 130; 131] and John Tang was the first, as far as I know, to record single channels from a number of mutants of ompF, probably >10 by now, and John is the only person to record single channels from maltoporin [189; 190], as far as I know, which he can now do routinely. While discussing John's skills, one should also point out that he recorded from both single Ca^{++} and single K^{+} channels *in intracellular membranes of sarcoplasmic reticulum of skinned muscle fibers* [191; 210].

To specifics, if we fail with ompF, we will look at closely related proteins that are known to have much smaller constriction zones and see how they behave in single channel recordings: which are suitable objects for IV measurements of selectivity? We will engineer mutants of suitable proteins to place (at least) 4 glutamates in a short length of their pore, and see if the mutants have the expected Ca^{++} selectivity. Good candidates for such a protein are maltoporin and omp32 which have much smaller constriction zones.

Constriction Site of Other Porins

	Structure	*Selectivity*	*Cross section area in A^2*
OmpF	trimer	cation	77
Omp32	trimer	anion	35
Maltoporin	trimer	sugars	24

Maltoporin lamB, a closely related but distinct molecule from ompF, specialized for sugar transport, has a much smaller pore, some 4 Å at its widest. We are looking carefully at the extensive literature on maltoporin to see if we should eventually use it as our main experimental object [14; 27; 35; 52; 63; 108; 109; 119; 126; 148; 149; 171-174; 190; 203; 204; 207; 213]. We already know how to make electrical measurements of single channel activity of maltoporin [190] and now know why such measurements have not been reported elsewhere (to the best of our knowledge): it requires particularly large (but brief) potential changes to initiate gating.

Omp32 is a natural anion selective porin which we ourselves have not yet handled, but which seems a suitable candidate [69; 216; 218] particularly because Karshikoff has performed an equilibrium electrostatic analysis showing why the protein has strong anion selectivity, even selectivity for divalent anions. We think a mutation of this selectivity to that of divalent cation selectivity would be particularly interesting.

Hemolysin has been extensively studied by our friends and colleagues Hagan Bayley and Orit Braha. In fact, we have just now (Sept 2004) been sent a sample and John Tang is starting experiments with it (in another project). I spent 12 days with Hagan and Orit in Oxford this year and see them often. We can get the adv… …ods to fi… …11-13; …with hem… …gan and…

Table 1: Comparison of permeation properties between L-Ca and EAE

	Net charge	Volume (nm^3)	g (pS)	P_{Ca}/P_{Na}	$0.5I_{Na}$
L-Ca	-4*e*	0.375	10	100	≈1 μM
EAE	-6*e*	2	158	3	>1 mM

Key to symbols: g is the conductance in $CaCl_2$; P_{Ca}/P_{Na} is the Ca^{++} over Na^+ permeability obtained from reversal potential measurements; $0.5I_{Na}$ is the Ca^{++} concentration needed to reduce the Na^+ current by 50%.

(I found it too difficult to reproducibly embed figures in text containing equations and references)

Table 2: Occupied space and their effect on pore volume of amino acids F, Y and W

Residue		Volume (nm^3)	SEA
Alanine	**A**	0.089	48/35
Cysteine	**C**	0.109	32/54
Arginine	**R**	0.173	84/5
Tryptophan	**W**	0.228	49/44

SEA=Solvent Exposed Area. For example, '48/35' indicates a 48% chance that the AA points into the polar solvent and 35% chance to find in buried in a low dielectric (protein or lipid) environment.

Fig . 4A **Fig. 4B**

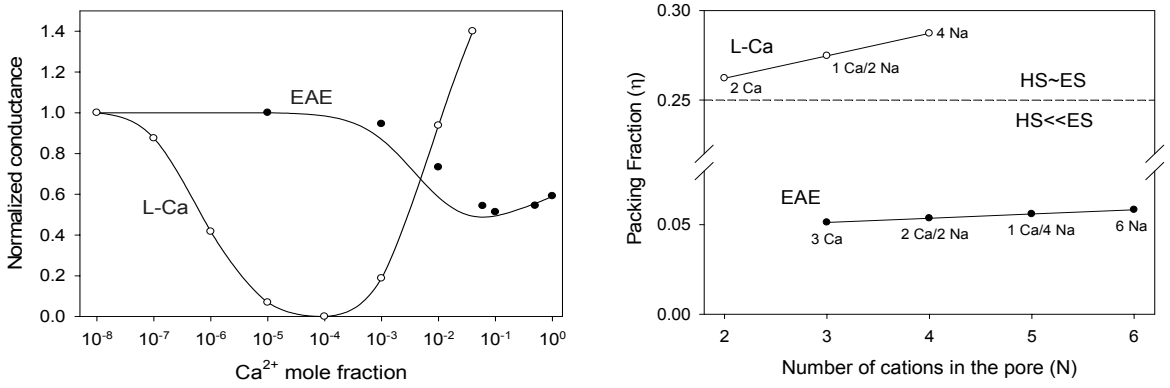


Fig 4A. Sensitivity of Na^+ current to mole fraction of Ca^{++} , in both L-type Ca-channel (L-Ca) and EAE mutant. Note that L-Ca is affected by a Ca^{++} concentration even in the nM range, whereas EAE requires Ca^{++} concentrations in the μM range.

Fig 4B. Comparison of packing fractions in L-Ca and EAE. In L-Ca, both electrostatics (ES) and crowding (HS) contribute to ion selectivity. In EAE crowding is not an issue because it is ~5 times bigger than L-Ca. The HS component is lacking, making the channel less selective.

Fig. 5. Contributions of charge and crowding to ion selectivity in *ompF*

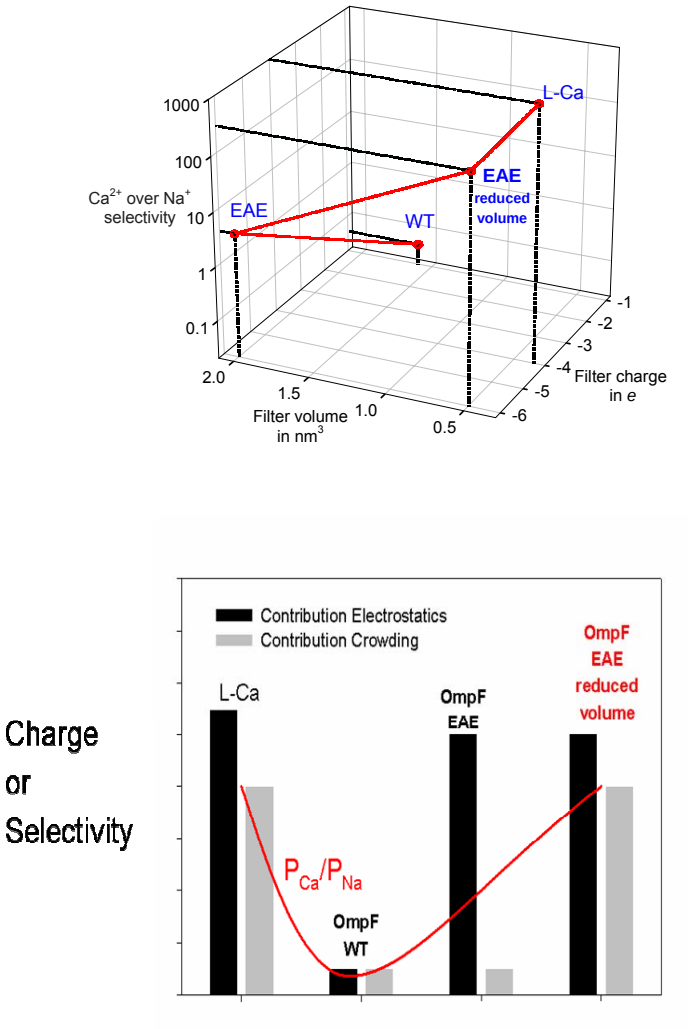


Fig. 6 Thiosulfonates. Examples of two negatively charged (A and B) and one neutral thiosulfonate (C) that can be used for the chemical modification of introduced cysteines in *ompF*. As indicated, the length of all three thiosulfonates is variable, a feature that significantly enhances the flexibility of this approach.

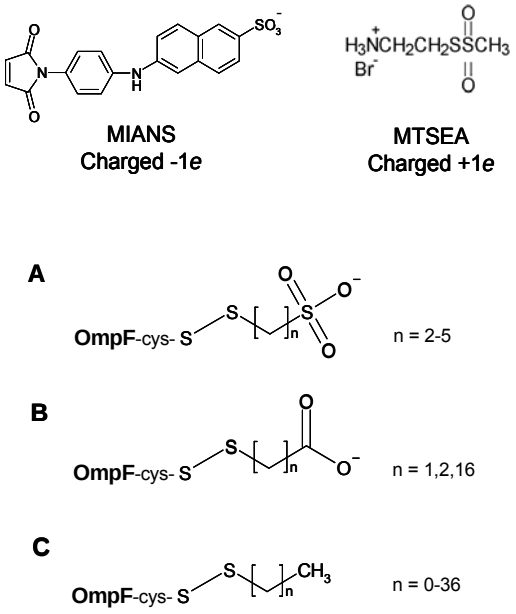
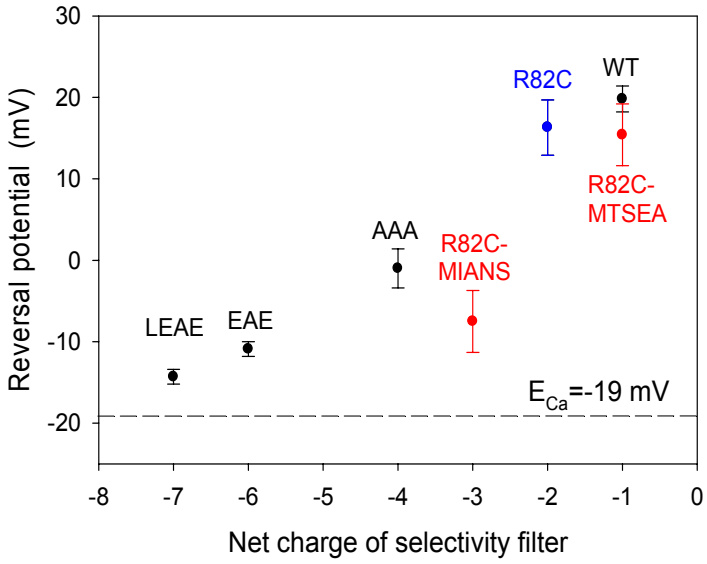


Fig. 7. **Reversal Potential and Net Charge** in Channels. Ca^{++} vs. Cl selectivity and the net charge accumulated in the selectivity filter of the pore in wild type WT and (chemically modified) mutant *ompF* protein. Reversal potentials were recorded in a 1/0.1 M $CaCl_2$ gradient, pH 7.4 and the Ca^{++} equilibrium potential E_{Ca}=–19 mV is shown. Mutant abbreviations used: AAA=R42A, R82A, R132A; EAE=R42E, R82A, R132E; LEAE=K16L, R42E, R82A, R132E. R82C labeling with MIANS and MTSEA was performed overnight.



References

1. Aboud S, Marreiro D, Saraniti M, Eisenberg R. 2004. A Poisson P3M Force Field Scheme for Particle-Based Simulations of Ionic Liquids. J. Computational Electronics, in the press.
2. Acker JP, Lu XM, Young V, Cheley S, Bayley H, Fowler A, Toner M. 2003. Measurement of trehalose loading of mammalian cells porated with a metal-actuated switchable pore. Biotechnol Bioeng 82(5):525-32.
3. Alberts B, Bray D, Johnson A, Lewis J, Raff M, Roberts K. 1998. Essential Cell Biology. New York: Garland. 630 p.
4. Allen R, Hansen J-P, Melchionna S. 2001. Electrostatic potential inside ionic solutions confined by dielectrics: a variational approach. Phys Chem Chem Physics 3:4177-4186.
5. Almers W, McCleskey EW. 1984. Non-selective conductance in calcium channels of frog muscle: calcium selectivity in a single-file pore. J.Physiol. 353:585-608.
6. Almers W, McCleskey EW, Palade PT. 1984. Non-selective cation conductance in frog muscle membrane blocked by micromolar external calcium ions. J. Physiol. 353:565-583.
7. Armstrong CM, Neyton J. 1992. Ion permeation through calcium channels. Ann. N Y Acad. Sci. 635:18-25.
8. Ashcroft FM. 1999. Ion Channels and Disease. New York: Academic Press. 481 p.
9. Barthel J, Buchner R, Münsterer M. 1995. Electrolyte Data Collection Vol. 12, Part 2: Dielectric Properties of Water and Aqueous Electrolyte Solutions. Frankfurt am Main: DECHEMA.
10. Barthel J, Krienke H, Kunz W. 1998. Physical Chemistry of Electrolyte Solutions: Modern Aspects. New York: Springer.
11. Bayley H, Cremer PS. 2001. Stochastic sensors inspired by biology. Nature 413(6852):226-30.
12. Bayley H, Jayasinghe L. 2004. Functional engineered channels and pores (Review). Mol Membr Biol 21(4):209-20.
13. Bayley H, Martin CR. 2000. Resistive-Pulse Sensing-From Microbes to Molecules. Chem Rev 100(7):2575-2594.
14. Benz R, Schmid A, Vos-Scheperkeuter GH. 1987. Mechanism of sugar transport through the sugar-specific LamB channel of Escherichia coli outer membrane. J Membr Biol 100(1):21-9.
15. Berry SR, Rice SA, Ross J. 2000. Physical Chemistry. New York: Oxford. 1064 p.
16. Binder K, editor. 1995. The Monte Carlo Method in Condensed Matter Physics. Second ed. New York: Springer.
17. Blum L. 1980. Simple electrolytes in the mean spherical approximation. In: Henderson DJ, Eyring H, editors. Theoretical Chemistry: Advances and Perspectives. New York: Academic Press.
18. Blum L. 1980. Solution of the Ornstein Zernike Equation for a mixture of hard ions and Yukawa Closure. Journal of Statistical Physics 22:661.
19. Blum L, Holovko MF, Protsykevych IA. 1996. A solution of the multiple-binding mean spherical approximation for ionic mixtures. J. Stat. Phys. 84(1/2):191–203.
20. Blum L, Vericat F, Degreve L. 1999. The Multiyukawa model of water. Physica A 265:396.
21. Boda D, Busath DD, Eisenberg B, Henderson D, Nonner W. 2002. Monte Carlo simulations of ion selectivity in a biological Na+ channel: charge-space competition. Physical Chemistry Chemical Physics (PCCP) 4:5154-5160.
22. Boda D, Busath DD, Henderson D, Sokolowski S. 2000. Monte Carlo Simulations of the Mechanism of Channel Selectivity: the competition between Volume Exclusion and Charge Neutrality. Journal of Physical Chemistry B *104*:8903-8910.

23. Boda D, Gillespie D, Nonner W, Henderson D, Eisenberg B. 2004. Computing induced charges in inhomogeneous dielectric media: application in a Monte Carlo simulation of complex ionic systems. Phys Rev E Stat Nonlin Soft Matter Phys 69(4 Pt 2):046702.
24. Boda D, Henderson D, Busath DD. 2001. Monte Carlo Study of the Effect of Ion and Channel Size on the Selectivity of a Model Calcium Channel. Journal of Physical Chemistry B 105(47):11574-11577.
25. Boda D, Henderson D, Chan K-Y. 1999. Monte Carlo study of the capacitance of the double layer in a model molten salt. Journal of Chemical Physics 110:5346-5350.
26. Boda D, Henderson D, Patrykiejew A, Sokolowski S. 2001. Density Functional Study of a Simple Membrane Using the Solvent Primitive Model. J Colloid Interface Sci 239(2):432-439.
27. Boos W, Shuman H. 1998. Maltose/maltodextrin system of Escherichia coli: transport, metabolism, and regulation. Microbiol Mol Biol Rev 62(1):204-29.
28. Boulous MI, Fauchais P, Pfender E. 1994. Thermal Plasmas. New York: Plenum. 452 p.
29. Braha O, Gu LQ, Zhou L, Lu X, Cheley S, Bayley H. 2000. Simultaneous stochastic sensing of divalent metal ions. Nat Biotechnol 18(9):1005-7.
30. Briggs WL, Henson VE. 1995. The DFT: An Owner's Manual for the Discrete Fourier Transform. Philadelphia: SIAM. 434 p.
31. Brigham EO. 1988. Fast Fourier Transform and its Applications. Englewood Cliffs, NJ: Prentice Hall. 448 p.
32. Cardenas AE, Coalson RD, Kurnikova MG. 2000. Three-Dimensional Poisson-Nernst-Planck Studies. Influence of membrane electrostatics on Gramicidin A Channel Conductance. Biophysical Journal 79.
33. Catacuzzeno L, Nonner W, Blum L, Eisenberg B. 1999. Ca Selectivity in the 'EEEE' Locus of L-type Ca Channels. Biophysical Journal 76:A259.
34. Catacuzzeno L, Nonner W, Eisenberg B. 1999. PNP2 Links Crystallographic Structure and Conduction in K Channels. Biophysical Journal 76:A79.
35. Charbit A. 2003. Maltodextrin transport through lamb. Front Biosci 8:s265-74.
36. Chazalviel J-N. 1999. Coulomb Screening by Mobile Charges. New York: Birkhäuser. 355 p.
37. Chen D, Xu L, Tripathy A, Meissner G, Eisenberg R. 1997. Rate Constants in Channology. Biophys. J. 73(3):1349-1354.
38. Chhih A, Bernard O, Barthel JMG, Blum L. 1994. Transport Coefficients and Apparent Charges of Concentrated Electrolyte Solutions: Equations for Practical Use. Ber. Bunsenges. Phys. Chem. 98:1516-1525.
39. Conway BE. 1969. Electrochemical Data. Westport CT USA: Greenwood Press Publishers. 374 p.
40. Corry B, Allen T, Kuyucak S, Chung S. 2000. A model of calcium channels. Biochim Biophys Acta 1509(1-2)(.):1-6.
41. Corry B, Allen T, Kuyucak S, Chung S. 2001. Mechanisms of permeation and selectivity in calcium channels. Biophys J 80:195-214.
42. Corry B, Allen TW, Kuyucak S, Chung SH. 2000. A model of calcium channels. Biochim Biophys Acta 1509(1-2):1-6.
43. Corry B, Allen TW, Kuyucak S, Chung SH. 2001. Mechanisms of permeation and selectivity in calcium channels. Biophys J 80(1):195-214.
44. Corry B, Kuyucak S, Chung SH. 2000. Tests of continuum theories as models of ion channels. II. Poisson-Nernst-Planck theory versus brownian dynamics. Biophys J 78(5):2364-81.
45. Corry B, Kuyucak S, Chung SH. 2003. Dielectric self-energy in poisson-boltzmann and poisson-nernst-planck models of ion channels. Biophys J 84(6):3594-606.

46. Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, Jansonius JN, Rosenbusch JP. 1992. Crystal structures explain functional properties of two E coli porins. Nature 358:727-733.
47. Damocles. 1999. *web address:* http://www.research.ibm.com/DAMOCLES/ref_intro.html.
48. Davis HT. 1996. Statistical Mechanics of Phases, Interfaces, and Thin Films. New York: Wiley-VCH. 712 p.
49. Dixon M, Webb EC. 1979. Enzymes. New York: Academic Press. 1116 p.
50. Durand-Vidal S, Simonin J-P, Turq P. 2000. Electrolytes at Interfaces. Boston: Kluwer.
51. Durand-Vidal S, Turq P, Bernard O, Treiner C, Blum L. 1996. New Perspectives in Transport Phenomena in electrolytes. Physica A 231:123-143.
52. Dutzler R, Wang YF, Rizkallah P, Rosenbusch JP, Schirmer T. 1996. Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. Structure 4(2):127-34.
53. Edwards S, Corry B, Kuyucak S, Chung SH. 2002. Continuum electrostatics fails to describe ion permeation in the gramicidin channel. Biophys J 83(3):1348-60.
54. Eisenberg B. 2000. Permeation as a Diffusion Process. In: DeFelice LJ, editor. Biophysics Textbook On Line "Channels, Receptors, and Transporters" http://www.biophysics.org/btol/channel.html#5.
55. Eisenberg B. 2003. Proteins, Channels, and Crowded Ions. Biophysical Chemistry 100:507 - 517.
56. Eisenberg RS. 1990. Channels as enzymes. J. Memb. Biol. 115:1–12.
57. Eisenberg RS. 1996. Atomic Biology, Electrostatics and Ionic Channels. In: Elber R, editor. New Developments and Theoretical Studies of Proteins. Philadelphia: World Scientific. p 269-357.
58. Eisenberg RS. 1996. Computing the field in proteins and channels. J. Membrane Biol. 150:1–25.
59. Eisenberg RS. 1999. From Structure to Function in Open Ionic Channels. Journal of Membrane Biology 171:1-24.
60. Eisenberg RS, Mathias RT. 1980. Structural analysis of electrical properties of cells and tissues. CRC Crit Rev Bioeng 4(3):203-32.
61. Ellinor PT, Yang J, Sather WA, Zhang J-F, Tsien R. 1995. Ca2+ channel selectivity at a single locus for high-affinity Ca2+ interactions. Neuron 15:1121-1132.
62. Fawcett WR. 2004. Liquids, Solutions, and Interfaces: From Classical Macroscopic Descriptions to Modern Microscopic Details. New York: Oxford University Press. 621 p.
63. Ferenci T. 1989. Selectivity in solute transport: binding sites and channel structure in maltoporin and other bacterial sugar transport proteins. Bioessays 10(1):3-7.
64. Feynman RP, Leighton RB, Sands M. 1963. The Feynman: Lectures on Physics, Mainly Electromagnetism and Matter. New York: Addison-Wesley Publishing Co.
65. Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422(6930):442-6.
66. Friedman HL. 1985. **A Course in Statistical Mechanics**. Englewood Cliffs, New Jersey: Prentice Hall.
67. Friel DD, Tsien RW. 1989. Voltage-gated calcium channels: direct observation of the anomalous mole fraction effect at the single-channel level. Proceedings of the National Academy of Sciences 86:5207-5211.
68. Frink LJD, Salinger AG. 1999. Density Functional Theory for Classical Fluids at Complex Interfaces. Albuquerque New Mexico: Sandia National Laboratory. Report nr SAND98-2840. 1-77 p.
69. Gerbl-Rieger S, Engelhardt H, Peters J, Kehl M, Lottspeich F, Baumeister W. 1992. Topology of the anion-selective porin Omp32 from Comamonas acidovorans. J Struct Biol 108(1):14-24.

70. Gillespie D, Eisenberg RS. 2002. Physical descriptions of experimental selectivity measurements in ion channels. European Biophysics Journal 31:454-466.
71. Gillespie D, Nonner W, Eisenberg RS. 2002. Coupling Poisson-Nernst-Planck and Density Functional Theory to Calculate Ion Flux. Journal of Physics (Condensed Matter) 14:12129-12145.
72. Gillespie D, Nonner W, Eisenberg RS. 2003. Density functional theory of charged, hard-sphere fluids. Phys Rev E 68:0313503 1-10.
73. Gillespie D, Nonner W, Henderson D, Eisenberg RS. 2002. A physical mechanism for large-ion selectivity of ion channels. Physical Chemistry Chemical Physics 4:4763-4769.
74. Grabert H, Devoret MH, editors. 1992. Single Charge Tunneling: Coulomb Blockade Phenomena in Nanostructures. New York: Plenum.
75. Graf P, Nitzan A, Kurnikova MG, Coalson RD. 2000. A dynamic lattice Monte Carlo model of ion transport in inhomogeneous dielectric environments: method and implementation. Journal of Physical Chemistry B 104:12324-12338.
76. Groetsch CW. 1999. Inverse Problems. Washington, DC: Mathematical Association of America. 222 p.
77. Gu LQ, Braha O, Conlan S, Cheley S, Bayley H. 1999. Stochastic sensing of organic analytes by a pore-forming protein containing a molecular adapter. Nature 398(6729):686-90.
78. Gu LQ, Cheley S, Bayley H. 2001. Capture of a single molecule in a nanocavity. Science 291(5504):636-40.
79. Gu LQ, Cheley S, Bayley H. 2001. Prolonged residence time of a noncovalent molecular adapter, beta-cyclodextrin, within the lumen of mutant alpha-hemolysin pores. J Gen Physiol 118(5):481-94.
80. Gu LQ, Cheley S, Bayley H. 2003. Electroosmotic enhancement of the binding of a neutral molecule to a transmembrane pore. Proc Natl Acad Sci U S A 100(26):15498-503.
81. Harned HS, Owen BB. 1958. The Physical Chemistry of Electrolytic Solutions. New York: Reinhold Publishing Corporation.
82. Henderson D. 1983. Recent Progress in the Theory of the Electric Double Layer. Progress in Surface Science 13:197-224.
83. Henderson D, Blum L, Lebowitz JL. 1979. An exact formula for the contact value of the density profile of a system of charged hard spheres near a charged wall. J. Electronal. Chem. 102:315-319.
84. Henderson D, Blum L, Noworyta JP. 1995. Inverse temperature expansion of some parameters arising from the solution of the mean spherical approximation integral equation for a Yukawa fluid. J. Chem. Phys. 102:4973-4975.
85. Henderson JR. 1992. Statistical Mechanical Sum Rules. In: Henderson D, editor. Fundamentals of Inhomogeneous Fluids. New York: Marcel Dekker. p 23-84.
86. Herrera JH, Blum L, Garcia-Llanos E. 1996. Thermodynamic properties of an asymmetric fluid mixture with Yukawa interaction in the mean spherical approximation. J Chem Phys 105:9288-9291.
87. Herrera JH, Ruiz-Estrada H, Blum L. 1996. Equation of state for a Yukawa fluid in the mean spherical approximation. J. Chem. Phys. 104:6327-6329.
88. Hess K. 1991. Monte Carlo Device Simulation: Full Band and Beyond. Boston, MA USA: Kluwer. 310 p.
89. Hess K. 2000. Advanced Theory of Semiconductor Devices. New York: IEEE Press. 350 p.
90. Hess K, Leburton JP, Ravaioli U. 1991. Computational Electronics: Semiconductor Transport and Device Simulation. Boston, MA USA: Kluwer. 268 p.
91. Hess P. 1988. Elementary properties of cardiac calcium channels: a brief review. Can J Physiol Pharmacol 66(9):1218-23.
92. Hess P, Lansman JF, Tsien RW. 1986. Calcium channel selectivity for divalent and monovalent cations. J Gen Physiol 88:293-319.

93. Hess P, Tsien RW. 1984. Mechanism of ion permeation through calcium channels. Nature 309:453-456.
94. Hille B. 2001. Ionic Channels of Excitable Membranes. Sunderland: Sinauer Associates Inc. 1-814. p.
95. Hockney RW, Eastwood JW. 1981. Computer Simulation Using Particles. New York: McGraw-Hill.
96. Hovarth AL. 1985. Handbook of Aqueous Electrolyte Solutions. New York: John Wiley & Sons. 622 p.
97. Howorka S, Bayley H. 2002. Probing distance and electrical potential within a protein pore with tethered DNA. Biophys J 83(6):3202-10.
98. Howorka S, Nam J, Bayley H, Kahne D. 2004. Stochastic detection of monovalent and bivalent protein-ligand interactions. Angew Chem Int Ed Engl 43(7):842-6.
99. Ichimura S. 1992. Statistical Plasma Physics. Basic Principles. New York: Addison-Wesley. 384 p.
100. Ichimura S. 1994. Statistical Plasma Physics. Condensed Plasmas. New York: Addison-Wesley. 289 p.
101. Im W, Roux B. 2002. Ion permeation and selectivity of OmpF porin: a theoretical study based on molecular dynamics, Brownian dynamics, and continuum electrodiffusion theory. J Mol Biol 322(4):851-69.
102. Im W, Roux B. 2002. Ions and counterions in a biological channel: a molecular dynamics simulation of OmpF porin from Escherichia coli in an explicit membrane with 1 M KCl aqueous salt solution. J Mol Biol 319(5):1177-97.
103. Jacoboni C, Lugli P. 1989. The Monte Carlo Method for Semiconductor Device Simulation. New York: Springer Verlag. pp. 1-356 p.
104. Jeanteur D, Schirmer T, Fourel D, Imonet V, Rummel G, Widmer C, Rosenbusch JP, Pattus F, Pages JM. 1994. Structural and functional alterations of a colicin-resistant mutant of ompF porin from *Eschericia coli*. Proc. Natl. Acad. Sci. USA 91:10675-10679.
105. Jencks WP. 1987. Catalysis in Chemistry and Enzymology. New York City: Dover. 836 p.
106. Kalyuzhnyi YK, Blum L, Reiscic J, Stell G. 2000. Solution of the associative mean spherical approximation for a multicomponent dimerizing hard-sphere multi-Yukawa fluid. J. Chem. Phys. 113:1135-1142.
107. Karlin A, Akabas MH. 1998. Substituted-cysteine accessibility method. Methods Enzymol 293:123-45.
108. Klebba PE, Hofnung M, Charbit A. 1994. A model of maltodextrin transport through the sugar-specific porin, LamB, based on deletion analysis. Embo J 13(19):4670-5.
109. Kullman L, Winterhalter M, Bezrukov SM. 2002. Transport of maltodextrins through maltoporin: a single-channel study. Biophys J 82(2):803-12.
110. Kuo C-C, Hess P. 1992. Characterization of the high-affinity Ca2+ binding sites in the L-type Ca2+ channel pore in rat phaeochromcytoma cells. Journal of General Physiology 466:657 - 682.
111. Kuo C-C, Hess P. 1992. Ion permeation through the L-type Ca2+ channel in rat phaeochromcytoma cells: two sets of ion binding sites in the pore. Journal of General Physiology 466:629-655.
112. Kurnikova MG, Coalson RD, Graf P, Nitzan A. 1999. A Lattice Relaxation Algorithm for 3D Poisson-Nernst-Planck Theory with Application to Ion Transport Through the Gramicidin A Channel. Biophysical Journal 76:642-656.
113. Lansman JB, Hess P, Tsien RW. 1986. Blockade of current through single calcium channels by Cd+2, Mg+2, and Ca+2. Voltage and concentration dependenceof calcium entry into the pore. Journal of General Physiology 88:321-347.
114. Lebowitz JL. 1964. Exact Solution of Generalized Percus-Yevick Equation for a Mixture of Hard Spheres. Physical Review 133A:895-899.
115. Lee KS, Tsien RW. 1984. High selectivity of calcium channels as determined by reversal potential measurements in single dialyzed heart cells of the guinea pig. Journal of Physiology (London) 354:253-272.

116. Levis RA, Mathias RT, Eisenberg RS. 1983. Electrical properties of sheep Purkinje strands. Electrical and chemical potentials in the clefts. Biophys J 44(2):225-48.
117. Likharev KK. 1999. Single-electron devices and their applications. Proceedings of the IEEE 87(4):606-632.
118. Lou K-L, Saint N, Prilipov A, Rummel G, Benson SA, Rosenbusch JP, Schirmer T. 1996. Structural and functional characterization of ompF porin mutants selected for large pore size. I. Crystallographic analysis. J. Biol. Chem. 271(34, August 23):20669-20675.
119. Lu D, Grayson P, Schulten K. 2003. Glycerol conductance and physical asymmetry of the Escherichia coli glycerol facilitator GlpF. Biophys J 85(5):2977-87.
120. Luchian T, Shin SH, Bayley H. 2003. Kinetics of a three-step reaction observed at the single-molecule level. Angew Chem Int Ed Engl 42(17):1926-9.
121. Luchian T, Shin SH, Bayley H. 2003. Single-molecule covalent chemistry with spatially separated reactants. Angew Chem Int Ed Engl 42(32):3766-71.
122. Lundstrom M. 2000. Fundamentals of Carrier Transport. NY: Addison-Wesley.
123. Martin PA. 1988. Sum Rules in Charged Fluids. Reviews of Modern Physics 60:1076-1127.
124. Mathias RT, Eisenberg RS, Valdiosera R. 1977. Electrical properties of frog skeletal muscle fibers interpreted with a mesh model of the tubular system. Biophys J 17(1):57-93.
125. Mathias RT, Rae JL, Eisenberg RS. 1979. Electrical properties of structural components of the crystalline lens. Biophys J 25(1):181-201.
126. Meyer JE, Schulz GE. 1997. Energy profile of maltooligosaccharide permeation through maltoporin as derived from the structure and from a statistical analysis of saccharide-protein interactions. Protein Sci 6(5):1084-91.
127. Miedema H, Bothwell JH, Brownlee C, Davies JM. 2001. Calcium uptake by plant cells--channels and pumps acting in concert. Trends Plant Sci 6(11):514-9.
128. Miedema H, de Boer AH, Pantoja O. 2003. The gating kinetics of the slow vacuolar channel. A novel mechanism for SV channel functioning? J Membr Biol 194(1):11-20.
129. Miedema H, Meter-Arkema A, Wierenga J, Tang J, Eisenberg B, Nonner W, Hektor H, Gillespie D, Wim Meijberg W. 2004. Permeation properties of an engineered bacterial OmpF porin containing the EEEE-locus of Ca2+ channels. Biophysical Journal, in the press.
130. Miedema H, Pantoja O. 2001. Anion modulation of the slowly activating vacuolar channel. J Membr Biol 183(2):137-45.
131. Miedema H, Romano LA, Assmann SM. 2000. Kinetic analysis of the K(+)-selective outward rectifier in Arabidopsis mesophyll cells: a comparison with other plant species. Plant Cell Physiol 41(2):209-17.
132. Miles G, Bayley H, Cheley S. 2002. Properties of Bacillus cereus hemolysin II: a heptameric transmembrane pore. Protein Sci 11(7):1813-24.
133. Miles G, Movileanu L, Bayley H. 2002. Subunit composition of a bicomponent toxin: staphylococcal leukocidin forms an octameric transmembrane pore. Protein Sci 11(4):894-902.
134. Movileanu L, Cheley S, Bayley H. 2003. Partitioning of individual flexible polymers into a nanoscopic protein pore. Biophys J 85(2):897-910.
135. Movileanu L, Cheley S, Howorka S, Braha O, Bayley H. 2001. Location of a constriction in the lumen of a transmembrane pore by targeted covalent attachment of polymer molecules. J Gen Physiol 117(3):239-52.
136. Moy G, Corry B, Kuyucak S, Chung S. 2000. Tests of continuum theories as models of ion channels. I. Poisson-Boltzmann theory versus
Brownian dynamics. Biophys J. 78(2349-2363).

137. Moy G, Corry B, Kuyucak S, Chung SH. 2000. Tests of continuum theories as models of ion channels. I. Poisson-Boltzmann theory versus Brownian dynamics. Biophys J 78(5):2349-63.
138. Nadler B, Hollerbach U, Eisenberg RS. 2003. Dielectric boundary force and its crucial role in gramicidin. Phys Rev E Stat Nonlin Soft Matter Phys 68(2 Pt 1):021905.
139. Nestorovich EM, Rostovtseva TK, Bezrukov SM. 2003. Residue ionization and ion transport through OmpF channels. Biophys J 85(6):3718-29.
140. Nonner W, Catacuzzeno L, Eisenberg B. 2000. Binding and Selectivity in L-type Ca Channels: a Mean Spherical Approximation. Biophysical Journal 79:1976-1992.
141. Nonner W, Catacuzzeno L, Eisenberg B. 2000. Ionic selectivity in calcium channels. Biophysical Journal 78:A455.
142. Nonner W, Chen DP, Eisenberg B. 1998. Anomalous Mole Fraction Effect, Electrostatics, and Binding in Ionic Channels. Biophysical Journal 74:2327-2334.
143. Nonner W, Chen DP, Eisenberg B. 1999. Progress and Prospects in Permeation. Journal of General Physiology 113(June):773-782.
144. Nonner W, Eisenberg B. 1998. Ion Permeation and Glutamate Residues Linked by Poisson-Nernst-Planck Theory in L-type Calcium Channels. Biophys. J. 75: 1287-1305.
145. Nonner W, Eisenberg B. 2000. Electrodiffusion in Ionic Channels of Biological Membranes. Journal of Molecular Fluids 87:149-162.
146. Nonner W, Gillespie D, Henderson D, Eisenberg B. 2001. Ion accumulation in a biological calcium channel: effects of solvent and confining pressure. J Physical Chemistry B 105:6427-6436.
147. Nonner W, L. , Catacuzzeno, Eisenberg B. 2000. Ionic selectivity in K channels. Biophysical Journal 78:A96.
148. Orlik F, Andersen C, Benz R. 2002. Site-directed mutagenesis of tyrosine 118 within the central constriction site of the LamB (Maltoporin) channel of Escherichia coli. I. Effect on ion transport. Biophys J 82(5):2466-75.
149. Orlik F, Andersen C, Benz R. 2002. Site-directed mutagenesis of tyrosine 118 within the central constriction site of the LamB (maltoporin) channel of Escherichia coli. II. Effect on maltose and maltooligosaccharide binding kinetics. Biophys J 83(1):309-21.
150. Parker DR, Norvell WA, Chaney RL. 1995. GEOCHEM-PC: A chemical speciation program for IBM and compatible personal computers. In: Chemical Equilibrium and Reaction Models. In: Loeppert RH, Schwab AP, Goldberg S, editors. Madison, Wisconsin, USA: Soil Science Society of America/American Society of Agronomy. p 253-269.
151. Peracchia C, editor. 1994. Handbook of Membrane Channels. New York: Academic Press. 580 p.
152. Philippsen A, Im W, Engel A, Schirmer T, Roux B, Muller DJ. 2002. Imaging the electrostatic potential of transmembrane channels: atomic probe microscopy of OmpF porin. Biophys J 82(3):1667-76.
153. Pietrobon D, Prod'hom B, Hess P. 1989. Interactions of protons with single open L-type calcium channels. Journal of General Physiology 94:1-21.
154. Pitzer KS. 1991. Activity Coefficients in Electrolyte Solutions. Boca Raton FL USA: CRC Press.
155. Prilipov A, Phale PS, Van Gelder P, Rosenbusch JP, Koebnik R. 1998. Coupling site-directed mutagenesis with high-level expression: largescale production of mutant porins from E. coli. FEMS Microbiology Letters 163:65-72.
156. Prod'hom B, Pietrobon D, Hess P. 1989. Interactions of protons with single open L-type calcium channels. Journal of General Physiology 94:23-42.
157. Pytkowicz RM. 1979. Activity Coefficients in Electrolyte Solutions. Boca Raton FL USA: CRC. 288 p.
158. Robinson RA, Stokes RH. 1959. Electrolyte Solutions. London: Butterworths Scientific Publications.

159. Rosenfeld Y. 1993. Free energy model for inhomogeneous fluid mixtures: Yukawa-charged hard spheres, general interactions, and plasmas. Journal of Chemical Physics 98(10):8126-8148.
160. Rosenfeld Y. 1993. Free-energy model for charged Yukawa mixtures: Asymptotic strong-coupling limit and a nonlinear mixing rule. Physical Review. E. Statistical Physics, Plasmas, Fluids, and Related Interdisciplinary Topics 47(4):2676-2682.
161. Rosenfeld Y. 2000. Excess-entropy and freezing-temperature scalings for transport coefficients: self-diffusion in yukawa systems. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics 62(5 Pt B):7524-7.
162. Rosenfeld Y, Blum L. 1986. Statistical Mechanics of Charged objects: General method and application to simple systems. J. Chem. Phys. 85:1556-1566.
163. Rosenfeld Y, Levesque D, Weis JJ. 1989. "Onsager-molecule" approach to liquid structure: The one-component plasma in two and three dimensions. Physical Review. A 39(6):3079-3087.
164. Rosenfeld Y, Schmidt M, Lowen H, Tarazona P. 1997. Fundamental-measure free-energy density functional for hard spheres: Dimensional crossover and freezing. Physical Review E 55(4 April):4245-4263.
165. Rothberg BS, Shin KS, Phale PS, Yellen G. 2002. Voltage-controlled gating at the intracellular entrance to a hyperpolarization-activated cation channel. J Gen Physiol 119(1):83-91.
166. Rowley RL. 1994. Statistical Mechanics for Thermophysical Calculations. Englewood Cliffs, NJ: PTR Prentice-Hall. 489 p.
167. Saint N, Lou K-L, Widmer C, Luckey M, Schirmer T, Rosenbusch JP. 1996. Structural and functional characterization of ompF porin mutants selected for large pore size. II. Functional characterization. J. Biol. Chem. 271(34, August 23):20676-20680.
168. Saint N, Prilipov A, Hardmeyer A, Lou K-L, Schirmer T, Rosenbusch J. 1996. Replacement of the sole hisdinyl residue in ompF porin from E. Coli by Threonine (H21T) does not affect channel structure and function. Biochem. and Biophys. Res. Comm. 223:118-122.
169. Sanchez-Quesada J, Saghatelian A, Cheley S, Bayley H, Ghadiri MR. 2004. Single DNA rotaxanes of a transmembrane pore protein. Angew Chem Int Ed Engl 43(23):3063-7.
170. Saraniti M, Aboud S, Eisenberg R. 2004. The Simulation of Ionic Charge Transport in Biological Ion Channels: an Introduction to Numerical Methods. Reviews in Computational Chemistry (in the press).
171. Schirmer RH, Keller T.A., Y.F. W, Rosenbusch JP. 1995. Structural basis for sugar translocation through maltoporin channels at 3.1Å resolution. Science 267:512-514.
172. Schirmer T. 1998. General and specific porins from bacterial outer membranes. Journal of Structural Biology 121:101-109.
173. Schirmer T, Cowan SW. 1993. Prediction of membrane-spanning beta-strands and its application to maltoporin. Protein Sci 2(8):1361-3.
174. Schirmer T, Keller TA, Wang YF, Rosenbusch JP. 1995. Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. Science 267(5197):512-4.
175. Schultz SG, Andreoli TE, Brown AM, Fambrough DM, Hoffman JF, Welsh JF. 1996. Molecular Biology of Membrane Disorders. New York: Plenum. 681 p.
176. Schuss Z, Nadler B, Eisenberg RS. 2001. Derivation of PNP Equations in Bath and Channel from a Molecular Model. Physical Review E 64:036116 1-14.
177. Schuss Z, Nadler B, Eisenberg RS. 2001. Derivation of Poisson and Nernst-Planck equations in a bath and channel from a molecular model. Phys Rev E Stat Nonlin Soft Matter Phys 64(3 Pt 2):036116.
178. Schutz CN, Warshel A. 2001. What are the dielectric "constants" of proteins and how to validate electrostatic models? Proteins 44(4):400-17.

179. Selberherr S. 1984. Analysis and Simulation of Semiconductor Devices. New York: Springer-Verlag. pp. 1-293. p.
180. Shin SH, Luchian T, Cheley S, Braha O, Bayley H. 2002. Kinetics of a reversible covalent-bond-forming reaction observed at the single-molecule level. Angew Chem Int Ed Engl 41(19):3707-9; 3523.
181. Simonin J-P. 1997. Real Ionic Solutions in the Mean Spherical Approximation. 2. Pure Strong Electrolytes up to Very High Concentrations and Mixtures, in the Primitive Model. Journal of Physical Chemistry B 101:4313-4320.
182. Simonin J-P, Bernard O, Blum L. 1998. Real Ionic Solutions in the Mean Spherical Approximation. 3. Osmotic and Activity Coefficients for Associating Electrolytes in the Primitive Model. Journal of Physical Chemistry B 102:4411-4417.
183. Simonin J-P, Bernard O, Blum L. 1999. Ionic Solutions in the Binding Mean Spherical Approximation. Thermodynamic Properties of Mixtures of Associating Electrolytes. Journal of Physical Chemistry B 103:699-704.
184. Simonin J-P, Blum L. 1996. Departures from ideality in pure ionic solutions using the mean spherical approximation. J Chem Soc, Faraday Transactions 92:1533-1536.
185. Simonin J-P, Blum L, Turq P. 1996. Real Ionic Solutions in the Mean Spherical Approximation. 1. Simple Salts in the Primitive Model. Journal of Physical Chemistry 100:7704-7709.
186. Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE. 1996. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science 274(5294):1859-66.
187. Suenaga A, Komeiji Y, Uebayasi M, Meguro T, Saito M, Yamato I. 1998. Computational observation of an ion permeation through a channel protein. Biosci Rep 18(1):39-48.
188. Sun ZP, Akabas MH, Goulding EH, Karlin A, Siegelbaum SA. 1996. Exposure of residues in the cyclic nucleotide-gated channel pore: P region structure and function in gating. Neuron 16(1):141-9.
189. Tang J, Chen D, Saint N, Rosenbusch J, Eisenberg R. 1997. Permeation through porin and its mutant G119D. Biophysical Journal 72:A108.
190. Tang J, Saint N, Rosenbusch J, Eisenberg R. 1997. Permeation through single channels of maltoporin. Biophysical Journal 72:A108.
191. Tang JM, Wang J, Eisenberg RS. 1989. K+-selective channel from sarcoplasmic reticulum of split lobster muscle fibers. J Gen Physiol 94(2):261-78.
192. Tang JM, Wang J, Eisenberg RS. 1992. Perfusing patch pipettes. Methods Enzymol 207:176-81.
193. Tang JM, Wang J, Eisenberg RS. 1992. Studies on intact sarcoplasmic reticulum: patch clamp recording and tension measurement in lobster split muscle fibers. Methods Enzymol 207:692-9.
194. Tang JM, Wang J, Quandt FN, Eisenberg RS. 1990. Perfusing pipettes. Pflugers Arch 416(3):347-50.
195. Tang Z, Scriven LE, Davis HT. 1994. Effects of solvent exclusion on the force between charged surfaces in electrolyte solution. J. Chem. Phys. 100:4527-4530.
196. Tang Z, Scriven LE, T. DH. 1992. A three-component model of the electrical double layer. J. Chem. Phys. 97:494-503.
197. Tata BV, Boda D, Henderson D, Nikolov A, Wasan DT. 2000. Structure of charged colloids under a wedge confinement. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics 62(3 Pt B):3875-81.
198. Triolo R, Blum L, Floriano MA. 1978. Simple electrolytes in the mean spherical approximation II: study of a refined model. Journal of Physical Chemistry 82:1368-1370.
199. Triolo R, Blum L, Floriano MA. 1978. Simple electrolytes in the mean spherical approximation III: a workable model for aqueous solutions. Journal of Chemical Physics 67:5956-5959.
200. Triolo R, Grigera JR, Blum L. 1976. Simple Electrolytes in the Mean Spherical Approximation. J. Phys. Chem. 80:1858-1861.

201. Tsien RW, Hess P, McCleskey EW, Rosenberg RL. 1987. Calcium channels: mechanisms of selectivity, permeation, and block. Annual Review of Biophysics and Biophysical Chemistry 16:265-290.
202. van der Straaten TA, Kathawala G, Trellakis A, Eisenberg RS, Ravaioli U. 2004. BioMOCA - a Boltzmann transport Monte Carlo model for ion channel simulation. Molecular Simulation (under review).
203. Van Gelder P, Dumas F, Bartoldus I, Saint N, Prilipov A, Winterhalter M, Wang Y, Philippsen A, Rosenbusch JP, Schirmer T. 2002. Sugar transport through maltoporin of Escherichia coli: role of the greasy slide. J Bacteriol 184(11):2994-9.
204. Van Gelder P, Dutzler R, Dumas F, Koebnik R, Schirmer T. 2001. Sucrose transport through maltoporin mutants of Escherichia coli. Protein Eng 14(11):943-8.
205. Varga S, Boda D, Henderson D, Sokolowski S. 2000. Density Functional Theory and the Capillary Evaporation of a Liquid in a Slit. J Colloid Interface Sci 227(1):223-226.
206. Varma S, Jakobsson E. 2004. Ionization states of residues in OmpF and mutants: effects of dielectric constant and interactions between residues. Biophys J 86(2):690-704.
207. Vogel H, Jahnig F. 1986. Models for the structure of outer-membrane proteins of Escherichia coli derived from raman spectroscopy and prediction methods. J Mol Biol 190(2):191-9.
208. Waisman E, Lebowitz JL. 1972. Mean Spherical Model Integral Equation for Charged Hard Spheres. I. Method of solution. Journal of Chemical Physics 56:3086-3093.
209. Waisman E, Lebowitz JL. 1972. Mean Spherical Model Integral Equation for Charged Hard Spheres. II. Spheres. Journal of Chemical Physics 56:3093-3099.
210. Wang J, Tang JM, Eisenberg RS. 1992. A calcium conducting channel akin to a calcium pump. J Membr Biol 130(2):163-81.
211. Wang MC, Collins RF, Ford RC, Berrow NS, Dolphin AC, Kitmitto A. 2004. The three-dimensional structure of the cardiac L-type voltage-gated calcium channel: comparison with the skeletal muscle form reveals a common architectural motif. J Biol Chem 279(8):7159-68.
212. Wang MC, Dolphin A, Kitmitto A. 2004. L-type voltage-gated calcium channels: understanding function through structure. FEBS Lett 564(3):245-50.
213. Wang YF, Dutzler R, Rizkallah PJ, Rosenbusch JP, Schirmer T. 1997. Channel specificity: structural basis for sugar discrimination and differential flux rates in maltoporin. J Mol Biol 272(1):56-63.
214. Yang J, Ellinor PT, Sather WA, Zhang JF, Tsien R. 1993. Molecular determinants of Ca2+ selectivity and ion permeation in L-type Ca2+ channels. Nature 366:158-161.
215. Yellen G. 1993. Calcium channels. Structure and selectivity. Nature 366(6451):109-10.
216. Zachariae U, Koumanov A, Engelhardt H, Karshikoff A. 2002. Electrostatic properties of the anion selective porin Omp32 from Delftia acidovorans and of the arginine cluster of bacterial porins. Protein Sci 11(6):1309-19.
217. Zematis JF, Jr., Clark DM, Rafal M, Scrivner NC. 1986. Handbook of Aqueous Electrolyte Thermodynamics. New York: Deisgn Institute for Physical Property Data, American Institute of Chemical Engineers.
218. Zeth K, Diederichs K, Welte W, Engelhardt H. 2000. Crystal structure of Omp32, the anion-selective porin from Comamonas acidovorans, in complex with a periplasmic peptide at 2.1 A resolution. Structure Fold Des 8(9):981-92.
219. Zhang L, Davis HT, White HS. 1993. Simulations of solvent effects on confined electrolytes. J. Chem. Phys. 98:5793-5799.

Section h. Consortium/Contractual Arrangements

Programmatic Arrangements.

We propose to model, simulate, construct and measure calcium binding sites in proteins. The PI himself is not trained in molecular biology and is fortunate to work with colleagues at BioMade Corporation/University of Groningen who are both trained and experienced in these techniques. The work is proposed to be done at BioMade because they can do what we cannot do at Rush, namely manipulate the genome and its products to make the proteins we wish to design and study.

I have worked with my close friend George Robillard, Professor and former Chair Dept of Biochemistry University of Groningen, and the founder and President of BioMade Corporation for some 13 years now, completing a number of DARPA funded projects together. George is an American citizen who has spent his entire career in Groningen, where he has had a distinguished career as a bench biochemist working with a wide variety of methods. George and his wife Valerie are close friends of my wife Ardyth and me and we have many reasons besides science to see each other and watch our families and children grow and prosper (and help each other with their problems, too).

Wim Meijberg is a senior scientist at BioMade who has worked with us continually for a decade or so, and Henk Meijberg is an experienced electrophysiologist [65; 127; 128; 130; 131] who has worked with us for nearly as long. Wim and Henk have visited Rush many times and have built their setup at BioMade with our advice and help, benefiting from John Tang's long experience and tremendous expertise in single channel measurements.

BioMade Corporation provides a nearly ideal environment for collaborative work like this. BioMade's facilities far exceed those available at Rush. BioMade has full access to the facilities at the University of Groningen, including all the expensive equipment **and supporting faculty and staff** with their experience and expertise necessary to manipulate the genome and its products. The workers at BioMade have full time available to research and follow the work ethic of their founder which is much more focused and intense than in typical universities in my experience.

The genetic manipulations etc we propose below are rather easy for BioMade and much less involved than in their other work (see http://www.biomade.nl/company.htm). In particular, BioMade is a world leader in the technologies of organogels, to prevent colloid and protein aggregation, hydrophobns to control attachment of proteins and permeability of protein films, patterning of polymers and proteins on solid surfaces with soft nanolithography, and preparation of polymer films for photovoltaic devices. All of these technologies are carried from exploratory laboratory research through development and implementation of actual commercial products at BioMade using their expertise and the resources of the University. These technologies are very much more involved than what we ask BioMade to do for us. It is easy for them (and wonderful for us) to help us make our molecules! **Preliminary work [129] shows that BioMade can make the mutants of ompF needed for this work and that we can make this collaboration work 'on the side' even without specific funding.**

Specifically,

1) BioMade will construct and test mutant proteins with the high densities of carboxyl groups and the small pore volumes needed to produce physiological Ca^{++} selectivity, using conventional site-directed mutagenesis. Amino acid(s) in the natural protein will be replaced with other amino acids with different size and chemical properties by modifying the genetic code from which the protein is made. BioMade will construct mutants with pores of small volume, ~200 $Å^3$. According to our models and simulations, selectivity requires very crowded charge, as found in many natural proteins, and so mutations introducing

amino acids with bulky side chains should reduce pore volume enough to produce biological selectivity of Ca^{++} *vs.* Na^{+}.

2) BioMade will also make chemical modifications of amino acids in the *ompF* protein using non-genetic methods to introduce new chemical groups into *ompF* mutants. BioMade will covalently link bulky chemical groups to amino acids of the protein, using well known methods of organic and bio- chemistry. Henk and Wim will introduce the natural amino acid cysteine that contains sulfhydryl groups to which bulky groups can be bound and link MTSEA or MIANS (defined in Fig. 6 of the Research Plan) to them. These bulky groups will produce the crowded charge in narrow pores (found in biological channels) needed to make highly selective channels.

Fiscal, and administrative arrangements

BioMade has had several subcontracts from Rush in the last decade and we and they are used to working with each other. Administrators of BioMade and of Rush are fully aware of the rules and regulations of both institutions and countries and have had no difficulty working within them or with each other. Rush University Medical Center requests 48% of the first $25,000 of consortium costs in year 1 only. This cost ($12,000) has been added to the modified total direct cost base for the initial funding period.

BioMade and Rush will gladly provide any further documentation of our customary fiscal and administrative arrangements requested by the NIH and of course can modify those arrangements as needed or requested.

Biomade Technology Foundation
Nijenborgh 4
9747 AG Groningen
The Netherlands

Tel. : +31 50 363 52 46
Fax : +31 50 363 44 29
E-mail: info@biomade.nl
Web : www.biomade.nl

BioMaDe
Bio-Organic Materials and Devices

Biomade Technology Foundation
Nijenborgh 4
9747 AG Groningen
The Netherlands

Groningen, September 20, 2004
Our reference: BM04-00-117GR/ib

STATEMENT OF INTENT TO ENTER INTO A CONSORTIUM AGREEMENT

Application Title:"Models and Simulations of Calcium Binding Sites"

Grantee Institution: Rush University Medical Center, Chicago, Il.

The appropriate programmatic and administrative personnel of each institution involved in the grant application are aware of the NIH consortium grant policy and are prepared to establish the necessary inter-institutional agreement consistent with that policy.

Principal Investigators: Dr. Robert S. Eisenberg

Co-Principal Investigator: Dr. W. Meijberg

Signature:

Prof. Dr. G. T. Robillard, Director
Biomade Technology Foundation

Dr. W. Meijberg
Chief Scientific Officer.

Principal Investigator/Program Director (last, First, Middle): Eisenberg, Robert S.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. _____ ☐ STTR Fast Track

☐ REVISION of application number: _____

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: _____

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl. and Phase II only)*

☐ No ☐ Previously reported

☐ Yes. If "Yes," ☐ Not previously reported

☐ SUPPLEMENT to grant number: _____

(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director: _____

☒ FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/ certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☒ DHHS Agreement dated: March 26, 2004 ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date _____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	267,172	x Rate applied	48.0	% = F&A costs	$	128,243
b. 02 year	Amount of base $	243,328	x Rate applied	48.0	% = F&A costs	$	116,798
c. 03 year	Amount of base $	250,730	x Rate applied	48.0	% = F&A costs	$	120,351
d. 04 year	Amount of base $	257,340	x Rate applied	48.0	% = F&A costs	$	123,523
e. 05 year	Amount of base $	264,662	x Rate applied	48.0	% = F&A costs	$	127,038
					TOTAL F&A Costs	$	615,953

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*

Principal Investigator/Program Director (last, First, Middle): Eisenberg, Robert S.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. _____ ☐ STTR Fast Track

☐ REVISION of application number: _____

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: _____

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl. and Phase II only)*

☐ No ☐ Previously reported

☐ Yes. If "Yes," ☐ Not previously reported

☐ SUPPLEMENT to grant number: _____

(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director: _____

☐ FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/ certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☐ DHHS Agreement dated: _____ ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☒ No DHHS Agreement, but rate established with Biomade Technology, The Netherlands Date _____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	124,400	x Rate applied	31.5	% = F&A costs	$	39,200
b. 02 year	Amount of base $	127,900	x Rate applied	31.5	% = F&A costs	$	40,300
c. 03 year	Amount of base $	131,600	x Rate applied	31.5	% = F&A costs	$	41,500
d. 04 year	Amount of base $	135,100	x Rate applied	31.5	% = F&A costs	$	42,600
e. 05 year	Amount of base $	138,700	x Rate applied	31.5	% = F&A costs	$	43,700
					TOTAL F&A Costs	$	207,300

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*

4. SMOKE-FREE WORKPLACE ☐ Yes ☒ No *(The response to this question has no impact on the review or funding of this application.)*

Prir