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.**

While much of the PI's previous research has been peripherally related to biomedicine, it was all done as an engineer's approach to solve biomedical problems. The materials were designed with only a basic understanding of the biological and medical needs. The proposed training opportunity would provide the PI with an in depth knowledge of some aspects of vascular medicine and physiology as well as added knowledge in mathematical modeling and peptide chemistry. *The overall goal is to expand the PI's experience and training in biomedicine as well as in peptide chemistry and modeling to position her for a lifetime of research in biomedicine*

Cardiovascular disease is the leading cause of death in the United States. The treatment of cardiovascular disease often involves surgically bypassing occluded segments of blood vessels with human saphenous vein grafts. The development of vein graft stenosis within 1 year after implantation occurs in up to 20-40% of grafts and frequently leads to end-organ failure, including myocardial infarction and extremity amputation. Short-term graft failure is due to technical problems and vasospasm during harvest and preparation. Long-term graft failure is due to a hyperplastic wound healing response, intimal hyperplasia. The purpose of this proposal is to develop a protein/ peptide based therapeutic agent to enhance graft patency.

The *hypotheses of this investigation* is that synthetic phosphorylated heat shock related protein human (HSP20) analogues (pHSP20) can be optimized and delivered in a controlled manner that will prevent vein graft spasm and intimal hyperplasia. The specific aims are to: 1) Optimize TAT-pHSP20 as a functionally active biomolecule. 1a) Develop and synthesize a panel of analogues of the TAT-pHSP20 peptides, 1b) Determine the bioactivity of the peptides *ex vivo* using strips of human saphenous vein grafts. 2) Develop and characterize controlled release systems for TAT-pHSP20 to ensure sustained delivery of the biomolecule in an effective therapeutic concentration. 2a) Determine the association and release of TAT from the heparin biogel. 2b) Determine the bioactivity of the biogel *ex vivo* in a muscle bath using strips of human saphenous vein grafts.

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

_____ Department of _____
_____ University _____
_____

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
_____	_____ University	Principal Investigator
_____	_____ University	Primary Mentor
_____	_____ University	Secondary Mentor
_____	University of _____	Secondary Mentor
_____	_____ University	Technician
Advisory Committee		
_____	_____ University	Advisory Committee
_____	_____ University	Advisory Committee
_____	_____ University	Advisory Committee
_____	_____ University	Advisory Committee

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

RCA TOC Substitute Page Candidate (Last, First, Middle):_____

Use this substitute page for the Table of Contents of Research Career Awards. The name of the candidate must be provided at the top of each printed page and each continuation page.

RESEARCH CAREER AWARD
TABLE OF CONTENTS
(Substitute Page)

Page Numbers

Note: Type density and size must conform to limits provided in the Specific Instructions.

*Include these items only when applicable.

CITIZENSHIIP

☒ U.S. citizen or noncitizen national

☐ Permanent resident of U.S. (If a permanent resident of the U.S., a notarized statement must be provided by the time of award.

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: PI's

The 1000 + sq. ft. laboratory contains an RC5Bplus centrifuge with SLA1500 and SS34 rotors, BioFlow III fermentor, Shimadzu BioSpec 1601 spectrophotometer, one regular and one gradient thermocycler, Flexi-dry lyophilizer, dissecting hood with Leica S6E microscope, Centrivap concentrator, egg incubator, MilliQ water system, and autoclave. There are 2 refrigerators, 3 freezers and a -80 ^{0}C freezer. Other equipment include a pH meter, top-loading and analytical balances, microbial incubator, microbial shaking incubator, 2 chemical hoods, electrophoresis equipment with visualization capabilities and a 4 ^{o}C walk-in room. The cell culture room contains all of the necessary equipment for mammalian cell culture including 2 Biosafety ClassII A/B cabinets, 2 CO_2 incubators, water bath, Nikon TS 100 microscope with Kodak digital camera and fluorescent capabilities. A TA instruments AR 1000 Rheometer and AKTA FPLC are also present.

Clinical:

Animal:

The Department of _____ runs its own ALAC accredited animal facility. It has large and small animal capabilities. A full time staff member, who has over 25 years of animal surgical experience, maintains the facility.

Computer:

There are 3 lab computers with zip drives, CD-ROM drives and 20 GB hard drives, and a Dell Inspiron 8000 laptop with 100 MB zip drive, DVD-ROM and 10 GB hard drive.

There are 2 office computers, which include a Dell Inspiron 4100 with floppy, Zip and DVD drives and an external 40 X CDRW. This computer also has a docking station. A second Dell Latitude with a Pentium 4 processor, 512 Mb Memory and a 30 Gb hard drive is also present in the PI's office.

Office: PI's

The office space is 12' X 14'. In addition to the computer equipment listed above, it contains an HP Scanner, an HP 2200 TN laser printer, phone and essential office furniture. The office has room for a small, 5 person meeting table.

Other:

_____ Center: The PI and the Sponsor also have a second laboratory called the _____ center, of which, the sponsor is director. This center is used by 4 PIs and includes: : 2 APEX 396 Peptide synthesizers, and AKTA FPLC Explorer, two Waters HPLCs, a high throughput Water HPLC, a Waters' capillary HPLC and Micromass Quadrupole-time of flight-hybrid mass spectrometer, and other support equipment for peptide synthesis, purification and characterization. Support equipment includes centrifuges, lyophilizers, refrigerators, freezers, etc.

The _____ Department operates three core research laboratories that support research in Molecular, Cell and Tissue Bioengineering which extends the PI's capabilities with additional cell culture, bioimaging and analytical equipment. The _____ **Facility** in 730 ft^2 of contiguous space houses an inverted microscope (Leica DM IRBE) with fluorescence, phase contrast, and modulation contrast capabilities, a Leica Axioplan with fluorescence, brightfield, and phase contrast capabilities, a Leica MZFLIII stereomicroscope with brightfield and fluorescence capabilities and micropippetor and micromanipulator system. There is a color digital video camera (Optronics 750D) and a digital (Optronics Magnafire) for fluorescence and bright field image acquisition and analysis. Computer capabilities for the Bioengineering Core Imaging include two Gateway Select 1000 (1 GHz) Systems for online image capture and processing and two Gateway Select 850 Systems for offline image processing and analyses. The _____ Facilities also house a goniometer with

computer imaging capabilities (Rame-Hart, Inc., Mountain Lakes, NJ) for surface analysis by contact angle measurements. _____ **Facility** has 1000 ft^2 with a main room reserved for standard cell culture. It contains four 4 foot biosafety cabinets, 4 CO_2 incubators, 2 microgravity bioreactors, 2 Nikon light microscopes with digital imaging and Cohu cameras, water baths, centrifuges, cryostorage vessel, refrigerator, 2 freezers and 1 -80 degree freezer. An adjoining room contains a pH meter, toploading and analytical balances, a UV/Vis spectrophotometer, microcentrifuge, a Beckman Optima Plus micro-ultracentrifuge with 8 ml and 2 ml tube capacity, a speed-vac unit, sonicator, a microbial incubator, a microbial shaking incubator and a lyophilizer. The third area is the viral room, which contains a 6-foot biosafety cabinet, a Nikon inverted microscope, 2 CO_2 incubators, and a water bath. The _____ **Facility** provides equipment for basic protein, DNA and polymer characterization. The facility includes HPLC and FPLC capabilities, a UV/Vis spectophotometer, Circular Dichroism, and a Raman spectrophotometer. It also includes 2 capillary electrophoresis systems, a thermocycler, a ELISA microplate reader with six filters, a fluorescent microplate reader, and microplate washers. There is also a goneometer with video imaging capabilities. In addition to the core analytical facility equipment, the protein chemistry laboratory has circular dichroism, peptide synthesis, peptide sequencing, MALDI-TOF mass spectroscopy and amino acid analysis. Balances and sonication equipment are also present.

_____ **Facilities** -- These facilities offer DNA and peptide synthesis (Cruachem PS 250 and Millipore 9050), DNA sequencing (ABI 377), protein sequencing (Porton 2090) and amino acid (Hewlett-Packard AminoQuant) analysis. In addition HPLC (Beckman 125) and capillary electrophoresis (Beckman P/ACE available for purification of macromolecules. Molecular weight determinations of proteins can be made by a Vestec matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS).

In the Dept. of _____ there is the _____ **facility,** a multidisciplinary shared resource which houses a Leica TCS NT upright and inverted scanning (4) laser microscope (visible and UV); video light microscopy; ratio-imaging fluorescence microscopy; atomic force microscopy; phosphorescence/ chemiluminescence/ radioisotope imaging; and computer-aided image processing. The Keck facility also has a Leica SP2 multiphoton scanning confocal microscope containing Ar 457. 488, 514 nm; Kr 568 nm; HeNe633 nm lasers and an IR spectrophysics Tsunami 8 watt pump laser that can be tuned to any excitation wavelength between 350 and 500 nm.

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

2 APEX 396 Peptide synthesizers located in P3 center
AKTA FPLC Explorer, two Waters HPLCs, a high throughput Water HPLC all located in the _____ center and used for peptide purification
a Waters' capillary HPLC and Micromass Quadrupole-time of flight-hybrid mass spectrometer located in the _____ center and used for peptide identification
Muscle Bath located in Dr. _____'s primary laboratory, used for vasorelaxation studies.

Section II: Specialized Information: Peptide-Based Therapeutics For Vascular Disease

1. Introduction to Revised Application

2. Letters of Reference

3. The Candidate

A. Candidate's Background

I am trained as a bioorganic polymer chemist and a chemical engineer. By combining expertise learned during my Ph.D. studies under _____ at the University of _____, _____ in the department of polymer science and engineering and those learned during my postdoctoral training at the University of _____ and the _____ _____ under _____, I have begun to develop biomimetic materials. These materials serve as a platform from which I am launching a career in biomedical research.

B. Career Goals and Objectives

My overall goal is to expand my experience and training in biomedicine as well as in peptide chemistry and modeling to position myself for a lifetime of research in biomedicine. To achieve these goals, I have worked closely with my mentor, _____, M.D. to develop a research training and educational plan that will catalyze my leap into the biomedical arena. Dr. _____ and I also have identified secondary mentors in the areas of biological process modeling, _____, Ph.D., and peptide chemistry, _____, Ph.D., to help guide me in these areas. *With guidance from my mentors, my goal for the next five years is to develop both optimized biomimetic peptides and drug delivery platforms to deliver peptide vascular therapeutics.*

The overall plan includes working closely with my mentors in research, but is broadened through attendance at conferences and short courses, a review of primary literature and formal coursework. Through a combination of hands-on activities and didactic training, I plan to build upon my quantitative and basic biological skills to become proficient and creative in the area of cardiovascular biomedicine. The plan that Dr. _____and I have developed combines, from the outset, building both research-based and classroom-based skills. The plan includes:

- Gain in-depth understanding of cell physiology and cardiovascular physiology, biology and pathology
- Expand skills in polymer chemistry to include peptide synthesis and structural analysis
- Use engineering skills to develop predictive mathematical models for peptide release from polysaccharide gels
- Develop feedback loops that allow optimization of the peptide-based therapeutics for function and optimal release from polysaccharide matrices
- Gain additional knowledge in the responsible conduct of research in biomedicine
- Translate research into the clinic through the advancement of intellectual property and technologic transfer

The ultimate goal of the training period is to provide me with the skills and knowledge to direct high-level biomedical research. I am currently directing research in the areas of materials inspired by biology. During the next five year, I will explore ways in which these biomimetic materials can be translated into useful extracellular matrices and drug delivery agents for cardiovascular therapeutics.

C. Career Development Activities during Award Period-Didactic Training

Coursework will be completed during the first two years of training, while attendance at national meetings will ramp up over the five-year period. The classroom training will be used to provide a solid foundation in the area of protein and peptide structure, _____: Biophysical Chemistry, and in cellular physiology _____ Cellular Physiology. Much of the material from class can be generalized to biomedicine and will allow expansion into other areas of biomedical research once the PI has achieved solid footing in the cardiovascular research area.

The conferences that will increase the PI's knowledge in vascular biology include: the annual FASEB, the annual Research Initiatives in Vascular Disease, and the Vascular Cell Biology Gordon Conference in 2005. In addition to the didactic training, the PI will gain knowledge of intellectual property protection and technologic transfer. The work to date has led to the development of an intellectual property platform. The PI and her mentor will work closely with the technology transfer office at _____ to advance the research into the clinic. This will allow the PI to gain relevant knowledge with respect to bench to bedside issues.

Research Activities: Research activities will include functional optimization, structural characterization and modeling of a peptidomimetics of the small heat shock-like protein HSP20. This will work be carried out with input from Dr. _____. While the initial work will be done in Dr. _____'s laboratory at the University of _____, all of the required equipment is available in the PI's laboratory or in core laboratories at _____ University. The techniques and knowledge gained under Dr. _____'s guidance is readily transferable to the PI's laboratory.

The development of the polysaccharide gels is ongoing in the PI's laboratory as is initial basic drug diffusion modeling. More complex modeling will be done in collaboration with Dr. _____. Modeling of the complex release profiles of therapeutic peptides from the polysaccharide gels will allow us to better engineer both the gels and the peptides to obtain required release profiles for the therapeutic peptides (release and transduction are coupled through heparin-binding ability) as described further in the experimental section.

Application of the therapeutic peptide and gel carrier to blood vessels and studies of the physiological affect of the therapeutics will be carried out under the guidance of Dr. _____. Experiments will be performed both to attempt to further understand and to modulate smooth muscle physiology and pathology.

Responsible Conduct of Research: *Education in the responsible conduct of biomedical research will be gained by taking a course offered through the biology department at _____ University. The course is a graduate course offered once a year, during the spring semester. The title of the course is "_____ Professional Values in Science" and topics covered in the course include: issues related to values in science such as collaboration, finances, legal issues, media, mentoring, ownership of ideas, and scientific integrity. This course will be taken during the first full spring of funding.*

Oversight of the Plan: The PI and primary mentor will have weekly meetings to assess the progress of the PI and to refine the program of study if it is deemed necessary. The advisory committee (consisting of Drs. _____, _____, and _____) and PI will meet quarterly to assess the PI's progress. They will also reevaluate the training program and make adjustments as required to ensure that by the end of the program, the PI is fully prepared to lead _____ own NIH funded research. Since Dr. _____ is in _____ and the remaining mentors are in _____, it may be necessary to hold some of these meeting via video conferencing. Both the University of _____ and _____ University are fully equipped to handle this. At least once a year, all of the mentors and the PI will meet in person.

Prior Experience: The PI has gained extensive training in chemical engineering and polymer science and engineering in a didactic sense. The candidate has also gained experience in polymer science and engineering in the research setting. Materials that assemble in controlled manners with controlled crosslink density/mechanical properties, growth factor release and cell adhesion have been synthesized and studied. Characterization of the materials included x-ray scattering, infrared spectroscopy, NMR spectroscopy, scanning electron microscopy, elemental analysis and rheology, among others. While much of the research has been peripherally related to biomedicine, it was all done as an engineer's approach to solve biomedical problems. The materials were designed with only a basic understanding of the biological and medical needs. The proposed training opportunity would provide the candidate with an in depth knowledge of some aspects of vascular biomedicine and physiology as well as added knowledge in modeling and peptide chemistry. These tools will be added to engineering and polymer chemistry tools to develop useful materials and therapeutics for cardiovascular medicine.

Prior research projects include synthesis and characterization of protein-polymers. Abstracts of some of the projects follow.

[Information redacted]

Work in the PI's laboratory now attempts to translate biological self-assembly into the world of synthetic polymers. By using motifs of biological molecules and linking these motifs to synthetic polymers, it is possible to control assembly of the synthetic polymers. Additional details on current work are given in the section on preliminary studies.

By the end of the training period, the PI will have extensive knowledge of vascular biomedicine, peptide synthesis and functional optimization including peptide modeling, and biological process modeling to compliment skills in polymer science and engineering. The ultimate goal of this project is to translate the research into the clinic.

4. Statement by Sponsor(s)

[Information redacted]

5. Environment and Institutional Commitment to the Candidate

A. Description of Institutional Environment

_____ University is an internationally recognized metropolitan Research I university serving approximately 50,000 students. The university is recognized nationally as a foremost research institution offering excellent undergraduate and graduate education. Approximately 3,600 undergraduates and 1,400 graduate students are pursuing engineering degrees at _____.

We define Bioengineering as the discipline of engineering that applies principles and methods from engineering, the physical sciences, the life sciences, and the medical sciences to understand, define, and solve problems in medicine, physiology, and biology. Our undergraduate and graduate programs educate students to use engineering principles and technology to develop instrumentation, materials, diagnostic and therapeutic devices, artificial organs, and other equipment needed in medicine and biology and to discover new fundamental principles regarding the functioning and structure of living systems. Faculty research focuses on these topics as well. The Bioengineering Program administers the Bachelor of Science in Engineering, Master of Science and Doctor of Philosophy degrees with the major Bioengineering. Currently, about 400 undergraduates and 80 graduate students are enrolled in these degree programs. The Bioengineering program includes an active post – doctoral training program and participates in a number of training grant programs that are available to our graduate students.

We classify the Bioengineering faculty into four groups: the core tenured or tenure-track Bioengineering faculty members, research faculty, affiliated faculty members from other _____ departments and a group of adjunct faulty members with primary appointments at other local institutions. Dr. _____ is one of fifteen full-time, tenured or tenure track faculty members that constitute the _____ faculty. Eight additional research faculty members, three affiliated and sixteen adjunct faculty members help the core tenure track faculty members administer the Bioengineering program. Details regarding the research interests of these faculty members can be found on the department's web site at _____. Additionally, one of our research faculty members, Mr. _____, a Senior Research Specialist, is responsible for overseeing our animal research program. Mr._____ assists the bioengineering faculty members in the design of animal protocols, trains faculty and staff in complex surgical procedures, and provides surgical expertise in support of animal research. The non-tenure track faculty members either collaborate with the core bioengineering faculty in research, educational, or training grants, or participate in the education of bioengineering graduate students.

The faculty is expected to obtain external research funding in the form of grants and contracts that can involve graduate students in the execution of their research. The faculty is expected to publish results of their research in archival journals and appropriate other forums. Within this context all of the core, research, affiliated and adjunct bioengineering faculty members are potential collaborators with Dr. _____ and relevant to the candidate's proposed development plan. The core faculty is listed in the following table.

Table 1. Core Bioengineering Faculty

Name, Highest Degree, Title	Research Interests
_____, Ph.D., Associate Professor of _____	Rehabilitation Engineering, Functional Neuromuscular Stimulation Systems, Neurophysiological Function in Spinal Cord Injury and Neural Network Control System Design
_____, Ph.D., Assistant Professor of _____	Tissue Engineering; Biomolecular Design, Synthesis and Assembly; Bioengineered Membrane Complexes
_____, Ph.D., Associate Professor of _____	Protein Purification, Acid/Base Molecular Interactions In Separations, Solid/Liquid Interfacial Phenomena, Scanning Probe Microscopy, Bio-Colloid Chemistry, Chromatography, Biosensor Immobilization.
_____, Ph.D., _____ Professor of _____ and Chair, _____ Department of _____	Development of Biosensors, Biomaterials, Biological Transport Phenomena, Physiological Systems Analysis and Simulation, Artificial Internal Organs
_____, Ph.D., Associate Professor of _____	Modeling And Simulation Of Physiological Systems, Dynamics And Control Of Neuromuscular Systems, Adaptive And Intelligent Control, Robotics, Biomechanics Of Posture And Movement, Neural Rehabilitation, Neural Prostheses And Functional Electrical Stimulation And Control
_____, Ph.D. Associate Professor of _____	Neural Engineering, Brain Dynamics
_____, Ph.D., Associate Professor of _____	Cell Interactions With Biomaterials, Surface Modifications For Enhanced Cell Attachment To Biomaterials, Vascular Graft/Stent Tissue Engineering, Tissue Engineered Intervertebral Disc Implants, Evaluation Of Cell Injury responses To Mechanical Stimuli
_____, Ph.D., Assistant Professor of _____	Neural Engineering, Microdevices
_____, Ph.D., Assistant Professor of _____	Biomaterials, Degradable And Biointeractive Materials, Bioorganic Polymer Chemistry, DNA Technology To Produce Novel Biofunctional Materials
_____, Ph.D., Associate Professor of _____	Molecular, Cell and Tissue Engineering, Artificial Organs, Biomaterials, Biosensors, Biotechnology, Bioseparations, Scanning Probe Microscopy, Fractals/Nonlinear Dynamical Systems
_____, Ph.D. Associate Professor of _____	Neurophysiological Control of Motor Systems (Sensorimotor Integration), Computational Neuroscience and Neurotrauma (Spinal Cord Injury)
_____, Ph.D., Associate Professor of _____	Neurostimulation, Skeletal Muscle Powered Cardiac Assistance, Cardiac Defibrillation, Applied Neural Control, Implantable Electrode Design, Physiology, Mathematical Modeling
_____, Ph.D., Professor of _____	Bioinstrumentation, Implantable Biochemical Sensors, Medical Ultrasound, Bioelectric Phenomena, Bioimpedance Imaging
_____, Ph.D., Assistant Professor of _____	Biomaterials, Drug Delivery
_____, Ph.D., Associate Professor Of _____	Biomechanics And Rehabilitation Engineering Design, Including Joint Mechanics, Computer Modeling Of Muscle, Tendon, And Joints, Optimal Control And Dynamic Analysis/Simulation Of Movement, Coordination And Functional Neuromuscular Stimulation

The quality of the Bioengineering faculty and their research productivity is high. Five Bioengineering faculty members have been recipients of _____ Research Grants. Bioengineering faculty research is supported by grants from the NIH, NSF, DARPA, The American Heart Association, and NASA. The Bioengineering Program received a _____ Award from the_____ Foundation to establish a Center for _____ at _____. The Center continues to exist with funding provided by the NIH and DARPA. The sponsored research

activities of the Core Bioengineering faculty members and the associated faculty members cover a broad range of bioengineering activities. Past faculty funding has included two NSF Career Awards, a NSF Young Investigator award, a NIH FIRST award, and a NSF Presidential Faculty Fellows Award, NIH CAREER Award, and two NSF IGERT awards.

Because of the strategic importance of Bioengineering, _____ with the aid of a $3,000,000 _____ Award is significantly enhancing the Bioengineering program. In July of 2001, _____ established a separate department of Bioengineering. As part of the commitment made to the _____ Foundation, _____ has hired seven new Bioengineering faculty members (including Dr._____). Five of these new faculty members have research interests in molecular, cell and tissue engineering, areas that support Dr. _____'s proposed research development program. With funds from the grant we have also developed new core research and educational laboratories for molecular, cell and tissue engineering and for neural engineering. We have also expanded our coursework offerings for graduate students in areas that support Dr. _____'s research development. A major goal of the _____ Foundation _____ Award is the establishment of new research collaborations with the _____, _____ Institute, The _____ Research Center and _____'s _____ Program and the University of _____. In total, _____ will spend in excess of $10,000,000 on Bioengineering program enhancement. Bioengineering is part of the College of Engineering and Applied Sciences _____. It is also part of the University's _____ that will spend in excess of $30,000,000 to develop new biomedical research initiatives. Development of _____'s new _____ Institute will include multiple new buildings for biomedical research. New laboratories supporting the research of Dr. _____ are planned for the first of these buildings which is scheduled for completion in two years. Space in this new building will also be set aside for Drs. _____ and _____. Space vacated by these individuals will be allocated to future planned bioengineering faculty hires.

The university currently assigns 21,176 ft^2 of space to Bioengineering as shown in Table 2.

Table 2. Bioengineering Space Inventory (units in square feet)

Location	Faculty Offices	Research Labs	Animal Facilities	Shared Admin. Space	Instr. Labs	Totals
_____ Center	0	0	0	2270	0	2270
_____ Center	1544	0	0	0	0	1544
_____ Building 2nd Floor	0	5034	0	0	0	5034
_____ Building 3rd Floor	0	4655	1114	0	1043	6812
_____ Building	786	4212	518	0	0	5516
Totals	**2330**	**13901**	**1632**	**2270**	**1043**	**21176**

Bioengineering space includes administrative offices (2,270 ft^2), faculty offices (2,330), a large instructional laboratory (1,043), faculty research laboratories (13,901 ft^2), and AAALAC approved animal surgical and animal care facilities (1632 ft^2). This space is distributed in four buildings, the _____ Building (_____), the _____ Center (_____), G-Wing of the _____ Center (_____) and the _____ Building (_____). The Bioengineering instructional laboratories and the Bioengineering faculty research laboratories are located in the_____. Bioengineering faculty offices are located in the _____ Center. The shared administrative space is located in the _____ of the _____Center. All of this space is located on the main _____ Campus. In anticipation of future program growth, a total of 4,212 ft^2 of research laboratory space, 786 ft^2 of office space and 518 ft^2 are assigned to Bioengineering at the _____, a short ten-minute drive north of _____'s main campus. The _____ was previously the _____ Hospital. The space allocated for Bioengineering at the _____ includes the previous hospital's operating room suite. The University is allocating an additional 12,000 square feet of new space to accommodate the enhancements associated with the _____ Foundation's _____ Award. Other facilities available to support Dr. _____'s development plan include _____ Facilities within the _____ Centers, the _____ Facility; _____ Facilities; The Center for_____ (_____) facilities; and facilities within The Center for _____ (_____).

B. Institutional Commitment to Candidate's Research Career Development

[Information redacted]

6. Research Plan

A. Statement of Hypothesis and Specific Aims

Cardiovascular disease is the leading cause of death in the United States. The treatment of cardiovascular disease often involves surgically bypassing occluded segments of blood vessels with human saphenous vein grafts. The development of vein graft stenosis within 1 year after implantation occurs in up to 20-40% of grafts and frequently leads to end-organ failure, including myocardial infarction and extremity amputation. (Spencer et al. 1971; Varty et al. 1993; Fitzgibbon et al. 1996) Short-term graft failure is due to technical problems and vasospasm during harvest and preparation. Long-term graft failure is due to a hyperplastic wound healing response, intimal hyperplasia. The purpose of this proposal is to develop a protein/ peptide based therapeutic agent to enhance graft patency.

The rationale of gene therapy is to produce a biologically active protein in a target cell population. However, a novel therapeutic approach is to directly transduce biologically active proteins or the respective biologically active domain into target cells. Three specific factors support a proteomic based therapeutic approach to vein graft spasm:

1. The recent identification of the biomolecule that prevents contraction (spasm) and intimal hyperplasia in human vein grafts: the phosphorylated heat shock related protein HSP20.
2. The recent identification of peptide-based molecules that introduce biologically active proteins into mammalian cells, such as the protein transducing peptide TAT.
3. The ability to target sustained release of TAT-derived biomolecules using heparin-based biogels.

The *hypotheses of this investigation* is that synthetic human HSP20 analogues (pHSP20) can be optimized and delivered in a controlled manner that will prevent vein graft spasm and intimal hyperplasia. This proposal will expand on my abilities as a polymer chemist and engineer to develop strategic proteomic-based therapeutics for clinical problems. The specific aims are to:

1. **Optimize TAT-pHSP20 as a functionally active biomolecule.**
    a. **Develop and synthesize a panel of analogues of the TAT-pHSP20 peptides**
    b. **Determine the bioactivity of the peptides *ex vivo* using strips of human saphenous vein grafts.**
2. **Develop and characterize controlled release systems for TAT-pHSP20 to ensure sustained delivery of the biomolecule in an effective therapeutic concentration.**
    a. **Determine the association and release of TAT from the heparin biogel.**
    b. **Determine the bioactivity of the biogel *ex vivo* in a muscle bath using strips of human saphenous vein grafts.**

The advantages of this approach are that novel molecules are engineered molecules, which incorporate protein transduction and biomimetic analogues of a physiologically relevant molecule, HSP20. This obviates the problems associated with gene therapy by directly introducing a mimetic of the active molecule at relevant concentrations. Biogels will be used to efficiently target the active molecules. This is possible in that vascular grafts are removed from the circulation and thus can be treated with the biomolecules *ex vivo* before implantation.

B. Background, Significance and Rational

Reconstructive cardiovascular surgery and vein graft spasm: One of the ideal conduits for peripheral vascular and coronary reconstruction is the greater saphenous vein. However, the surgical manipulation during harvest of the conduit leads to vasospasm (LoGerfo et al. 1984). This is an important cause of early graft failure and may contribute to long-term graft failure (Allaire and Clowes 19997). The exact

etiology of vasospasm is complex and most likely multifactorial. Vein graft spasm is most likely due to impaired relaxation of the smooth muscle. Trauma from the harvest itself can cause endothelial damage and the loss of endothelial-derived relaxing agents. Studies using saphenous vein segments in a muscle bath have shown that human saphenous vein smooth muscle pre-contracted with agonist, is uniquely refractory to active relaxation (Walsh et al. 1992).

The current therapeutic approach to vein graft spasm is to add a phosphodiesterase inhibitor (papaverine) in the vein harvest solution (LoGerfo et al. l984). This activates cyclic nucleotide dependent signaling pathways by preventing the breakdown of cyclic nucleotides. However, studies have demonstrated decreases in the expression of many of the downstream effectors of this pathway. For example, the expression of soluble guanylate cyclase is markedly reduced in aged rats (Chen et al. 2000) and most bypasses are performed in elderly patients. Thus, an approach which targets the downstream effector, HSP20, of this pathway may be more clinically relevant in that it obviates problems associated with alterations in the expression of proximal receptors and/or signaling molecules.

In many circumstances, surgeons use mechanical distension to break the spasm. This mechanical distension is associated with significant injury to the media of the vein graft (LoGerfo et al. 1984). This medial injury leads to vein graft stenoses through intimal hyperplasia and limits the long-term viability of the conduit (LoGerfo et al. 1984). Since coronary vein graft failure has been reported to be as high as 30-50% at 3-5 years it is likely that optimizing the graft during harvest and the early stages of implantation, will decrease both short- and long-term graft failure.

Intimal hyperplasia: The leading cause of vein graft failure is intimal hyperplasia (Davies et al. 1995) which is a complex process but primarily involves the vascular smooth muscle cells "response to injury." This response involves changes in smooth muscle cell phenotype resulting in cells that proliferate, migrate, and produce matrix proteins. (Engelse et al. 2002) This “hyperplastic” response narrows the lumen of the vessel and leads to graft failure. The biochemical events leading to cellular proliferation, migration, and ultimately intimal hyperplasia are poorly understood. While a number of mediators have been postulated, the role of nitric oxide has gained considerable interest. Nitric oxide is a potent activator of cGMP and has been demonstrated to inhibit proliferation, (Garg and Hassid 1989; Dubey et al. 1995) migration, (Dubey et al. 1995) and intimal hyperplasia. (Cable et al. 1999) Furthermore, previous investigations have shown that the addition of cyclic nucleotides or the inhibition of their breakdown results in decreased vascular smooth muscle cell proliferation, (Cornwell et al. 1994; Boerth et al. 1997; Yu et al. 1997) migration, (Itoh et al. 2001) and intimal hyperplasia. (Inoue et al. 2000) Additionally, vessels with intimal hyperplasia are known to express lower levels of cGMP than normal vessels. (Masuda et al. 1999; Beppu et al. 2002) Taken together, these findings support the role of cyclic nucleotide-dependent signaling pathways in the development of intimal hyperplasia.

Heat shock related protein-20 (HSP20): Blood vessels are comprised of two major cell types, endothelial cells and vascular smooth muscle cells. The discovery that endothelial cells are capable of producing a substance (nitric oxide, NO), which relaxes the underlying smooth muscle cells, has been the cornerstone of vascular biology. Nitric oxide is a cell permeable gas that interacts with a heme-containing moiety in the guanylyl cyclase enzyme leading to increases in intracellular cGMP. $\propto$-adrenergic agonists and prostacyclin are other agents which relax vascular smooth muscle by receptor mediated activation of adenylyl cyclase, leading to increases in cAMP. Cyclic GMP and cAMP activate cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) respectively (Figure 1). HSP20 has recently been identified as a substrate protein of both PKG and PKA. (Beall et al. 1997) Cyclic nucleotide-dependent relaxation and cyclic nucleotide-dependent inhibition of contraction are associated with increases in the phosphorylation of HSP20 (Beall et al. 1999; Wang et al. 1999; Woodrum et al. 1999; Rembold et al. 2000). HSP20 is not phosphorylated in a model of vasospasm, human umbilical artery smooth muscle (Berg et al. 1995). Umbilical smooth muscle is uniquely refractory to cyclic nucleotide-dependent relaxation (Berg et al. 1995); (Brophy et al. 1997). Endothelial-dependent vasodilation in isolated perfused carotid arteries is also associated with increases in the phosphorylation of HSP20 (Jerius et al. 1999). Thus, physiologic levels of NO released by the endothelium lead to detectable increases in the phosphorylation of HSP20. Increases in the phosphorylation of HSP20 are also associated with relaxation of another type of muscle, skeletal muscle (Wang et al. 1999). The introduction of a small peptide analogue of HSP20, containing the phosphorylated serine, into transiently

permeabilized smooth muscle cells inhibits contraction of the cells (Beall et al. 1999). These data suggest that an increase in the phosphorylation of HSP20 represents a common final event in which the cyclic nucleotide-dependent signaling pathways converge to induce relaxation. This pathway is of significant pharmacologic interest in that numerous drugs in clinical use activate various aspects of the pathway (Figure 1).

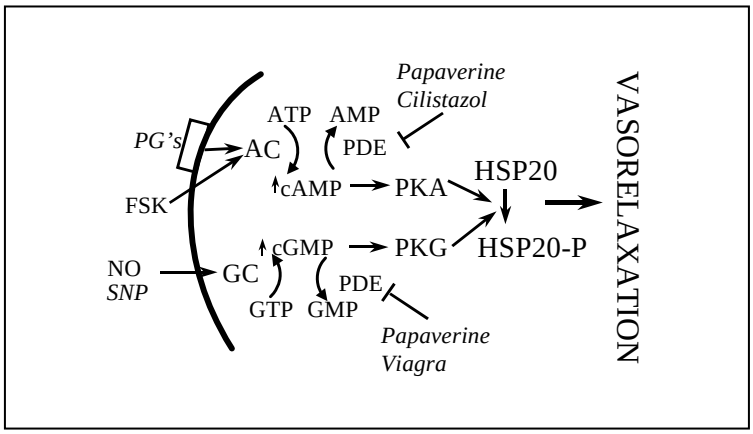

Figure 1. In a response to a variety of stimuli, endothelial cells (EC) produce nitric oxide (NO). NO is a gaseous molecule that diffuses across the cell membrane, activating guanylyl cyclase (GC) in the neighboring smooth muscle cells (SMC). This leads to increases in the cGMP and activation of the cGMP-dependent protein kinase (PKG). Forskolin (FSK) leads to relaxation via another cyclic nucleotide-dependent signaling pathway, activation of adenylyl cyclase (AC) and increases in cAMP. This leads to an activation of cAMP-dependent protein kinase (PKA). PKA and PKG phosphorylate HSP20 leading to vasorelaxation and stellation. Pharmacologic agents (italics) lead to vasorelaxation by activating adenylate cyclase (prostaglandins, PG), guanylate cyclase (sodium nitroprusside, SNP) or by inhibiting phophodiesterases (cilistazol, papaverine, viagra).

We have also determined that phenotypic modulation in rat aortic smooth muscle cells in culture is associated with a down regulation of both protein kinase G (PKG) and the small heat shock-related protein, HSP20. Smooth muscle cells that are stably transfected with PKG re-express both PKG and HSP20 (submitted for publication). These cells revert to a contractile phenotype, based on both biochemical and physiologic analyses. (Boerth et al. 1997) These data suggest that the expression of PKG, and the PKG substrate HSP20 are important in maintaining a contractile phenotype.

Protein based therapeutics: Previously, protein and peptide therapeutic approaches have targeted cells at the cellular membrane receptor level. (Ibrahim et al. 2000) In contrast, protein transduction allows intracellular delivery of proteins and offers significant advantages over gene transfection. TAT peptides and fusion proteins have been shown to transduce into cells in manner independent of cell surface receptors or active transport systems. (Schwarze et al. 1999; Wadia and Dowdy 2002) Uptake and internalization of TAT fusion peptides can be exploited as a powerful biotechnological tool for intracellular protein delivery. This allows for a proteomic based therapeutic approach that does not target receptors or signaling events, but rather downstream effector molecules. In the case of human saphenous vein grafts, this may be particularly relevant in that the expression of soluble guanylate cyclase is markedly reduced in aged vessels. (Chen and Cheung 1992) Thus, therapeutics targeted at upstream receptors would not be effective; yet, introducing the downstream effector molecule would be effective. This uptake and internalization of TAT can be exploited as a powerful biotechnological tool for intracellular protein delivery.

pHSP20 phosphopeptide analogues containing a protein transduction domain (PTD) have been synthesized. The application of these analogues to intact strips of vascular smooth muscle leads to dose dependent relaxation of the muscle (Figure 2). Finally, we have demonstrated that phosphorylated peptide analogues of HSP20 inhibit migration of cultured vascular smooth muscles and intimal hyperplasia in human saphenous vein grafts (see preliminary data). These data provide direct evidence that protein transduction can be used to specifically alter physiologic responses in vascular smooth muscle. They also suggest that small peptide domain sequences can mimic the effects of the entire HSP20 molecule.

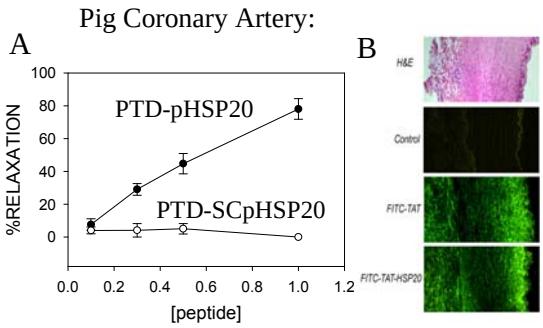

Figure 2: Phosphopeptide analogues of HSP20 relax pre-contracted rings of porcine coronary artery: Strips of porcine corotic artery were treated with seratonin and then chalanged with increasing amounts of the FITC-TAT-pHSP20 peptide analogue. Strips are observed to relax when treated with the active peptide (closed circles) but not with the scrambled peptide (open circles). Panel B shows, from top to bottem, a representative section of artery stained with H&E, no peptide added, TAT-FITC and TAT-pHSP20 FITC. Transduction of both TAT peptides can be seen clearly.

Taken together, these data suggest that HSP20 represents a target molecule that can be optimized to prevent both vein graft spasm and intimal hyperplasia. Increases in the phosphorylation of HSP20 represents the downstream effector molecule where the cyclic nucleotide dependent signaling pathways converge to induce vasorelaxation. In addition, these data suggest that protein transduction of biomimetic analogues of the phosphorylated HSP20 molecule have distinct biological activity. This information will be used to develop a proteomic based approach to enhance vein graft patency.

Delivery of Peptide Therapeutics: Many molecules are known to have the capacity to bind heparin. The most frequently discussed proteins are the family of heparin-binding growth factors including fibroblast growth factors and vascular endothelial growth factor (Lindner et al. 1992; Klagsbrun and Soker 1993). Proteins of the extracellular matrix also bind heparin including fibronectin and antithrombin III (ATIII) (Netzel-Arnett et al. 1991; Mardon and Grant 1994). The affinities of the ATIII heparin-binding domain and single amino acid mutants of the domain have been well characterized (Netzel-Arnett et al. 1991). The affinities can be changed over 13 fold. Many of the sequences of the heparin-binding domains and their relative affinity for heparin have been well defined (Table I). TAT has recently been described to be a heparin-binding peptide (Tyagi et al. 2001). The dissociation constant for TAT from heparin is related to the molecular weight of heparin and to the number of TAT molecules bound to an individual heparin molecule. For low molecular weight heparin and heparin to which only one molecule of TAT is bound, the Kd is approximately 0.7 μM (Rusnati et al. 1999). The Kd for TAT is approximately 30 nM when multiple TAT molecules are bound to one heparin molecule. While the former value is one order of magnitude lower than that of wild type antithrombin III (Table I), the later is two orders of magnitude higher. This suggests that the heparin binding properties of TAT and other transduction peptides can be used to develop release strategies from heparin-based biogels.

Table I. Heparin-binding peptides to be incorporated into the gels

Heparin-Binding Domain*	Affinity to Heparin	Relative Affinity to Heparin
KAFAKLAARLYRKA	High	100%
AAFAKLAARLYRKA	Medium	14.3%
TAT	Medium/Very High	14%/300%

***Underlined amino acids indicate those varied from wild type sequence**

Work in Dr. _____'s laboratory has demonstrated that the heparin-binding ability of TAT and other transduction peptides can be altered without significantly altering the peptides ability to cross cell membranes (Figure 3). We will use this information to develop methods of controlled release for peptide therapeutics. TAT, or modifications there of (PTD), will be released from the gel as a free peptide or as a free PTD-pHSP20. The heparin will remain bound to the gel due to the higher affinity for heparin of the heparin-binding peptides within the backbone of the gel compared to that of PTD. The free form of PTD or PTD-pHSP20 is the preferred form as it is the form most active in transduction (Tyagi et al. 2001).

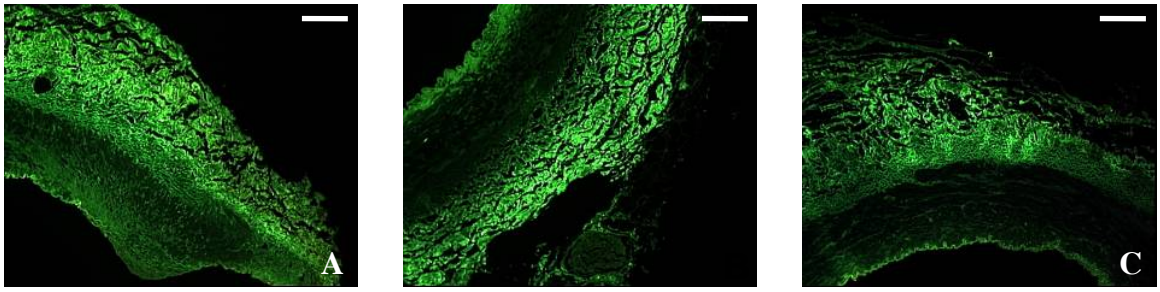

Figure 3. Pig coronary artery rings were placed in 2 ml HEPES buffer, pH 7.4. The tissue samples were incubated in the presence of 1 mM of the following peptides for 20 minutes at 37 °C: FITC-labeled PTD2 (A), FITC-labeled TAT (B) and FITC-labeled PTD3 (C). The artery rings were then washed three times with HEPES buffer, pH 7.4 and then fixed with 4% formalin in PBS. Images where collected using an inverted Leica DM IRB with attached Optronics Magnafire camera. FITC labels were excited at 490nm. All scale bars in the right column are 100 µm.

C. Preliminary Studies and Any Results

The data described below was generated under an STTR grant (_____) from the NIH. Funding from the STTR grant will end in August of 2003. Some of the work proposed here is a continuation of the work done under the STTR grant. Since the STTR funding will end prior to potential awarding of this proposal, there is no scientific overlap. In addition, funding was just awarded to the PI in the form of an NSF CAREER proposal from the Bioengineering directorate at the National Science Foundations. The CAREER award from the NSF does not support academic year salary for the PI, but it does support graduate and undergraduate student research. The subject matter of the CAREER award covers controlled release from polyethylene glycol star polymers and is not a direct overlap of the release studies discussed here. The CAREER award studies will complement studies in aim 2 since release from different polymers can be compared to one another.

Peptide analogues of HSP20 inhibit smooth muscle migration: A modified Boyden chamber assay was used to determine the effect of phosphopeptide analogues of HSP20 on smooth muscle migration. A10 smooth muscle cells were incubated in the presence of peptides containing the active phosphorylated sequence of HSP20 (TAT-pHSP20) or a scrambled series of the same amino acids (TAT-scHSP20). A concentration gradient of serum was developed within the chamber to induce to migration across the membrane. At the completion of the assay, migrated cells, in four high power fields, were counted under microscopy. The mean number of migrated cells for the basal (no FBS stimulation), FBS, TAT-scHSP20 + FBS, and TAT-pHSP20 + FBS groups were 61.3 $\pm$ 6.8, 322.3 $\pm$ 33.1, 229.0 $\pm$ 16.2, and 79.6 $\pm$ 14.8 respectively (Figure 4). A10 cells treated with FBS alone and TAT-scHSP20 + FBS showed significant increases in migration over the basal group ($P < 0.001$). In sharp contrast TAT-pHSP20 inhibited cellular migration by 75% compared to the FBS group ($P < 0.001$), and by 65% compared to TAT-scHSP20 + FBS treated cells ($P < 0.001$). Additionally, there was no significant difference in the number of migrated cells between the FBS and TAT-pHSP20 + FBS treated cells. These data suggest that phosphopeptide analogues of HSP20 inhibit cellular migration.

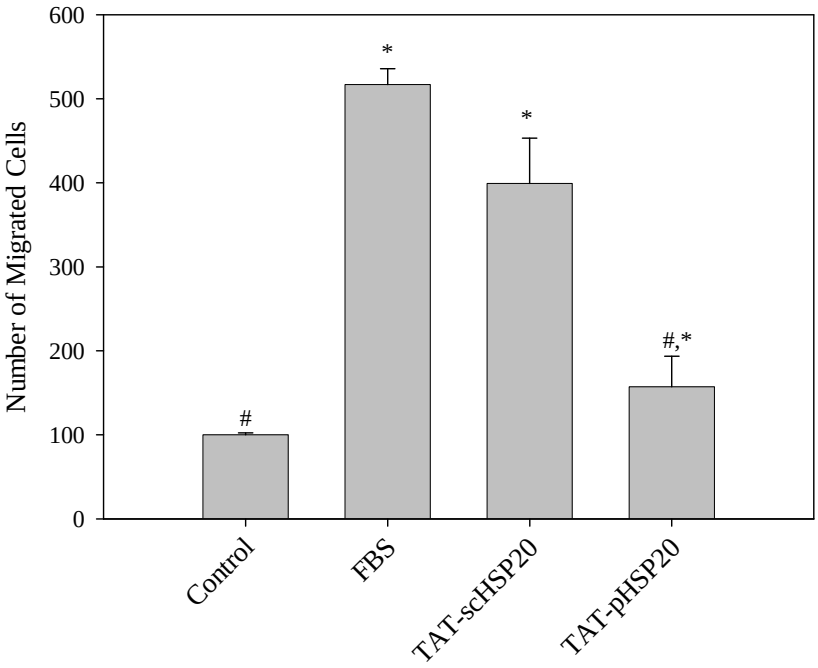

Figure 4. A10 cells were treated with 0.5% fetal bovine serum (FBS) alone, peptides containing the TAT transduction sequence linked to a scrambled amino acid sequence surrounding the HSP-20 phosphorylation site (TAT-scHSP20) + FBS, or the TAT transduction sequence linked to the amino acid sequence surrounding the HSP20 phosphorylation site (TAT-pHSP20) + FBS. Both of the HSP20 peptides contained a phosphoserine. Four separate experiments were performed. No significant difference was found between basal cells and TAT-pHSP20 + FBS treated cells (# $P > 0.05$). TAT-pHSP20 significantly inhibited migration over both FBS and TAT-scHSP20 + FBS treated cells (* $P < 0.05$)

The effect of phosphopeptide analogues of HSP20 on intimal hyperplasia in a human saphenous vein organ culture model: Six human saphenous veins, 36 rings total (12 control, 12 TAT-scHSP2, 12 TAT-pHSP20), were cultured for 14 days in the presence of 30% serum. All veins were deemed viable at the time of culture by adequate contraction with a serotonin challenge in a muscle bath. The average intimal thickness of the pre-cultured veins was 25.4 $\pm$ 7.8 μm. After culture the average intimal thickness of the control and TAT-scHSP20 treated veins was 58.4 $\pm$ 7.2 μm and 60.7 $\pm$ 8.5 μm while that of the TAT-pHSP20 treated veins was 24.7 $\pm$ 4.2 μm. The control and TAT-scHSP20 treated veins showed a 57% and 58% increase in intimal thickness compared to pre-culture ($P = 0.05$; $P = 0.04$ respectively). TAT-pHSP20 treated veins had an intimal thickness that was not significantly different compared to pre-cultured veins ($P = 1.00$) (Figure 5).

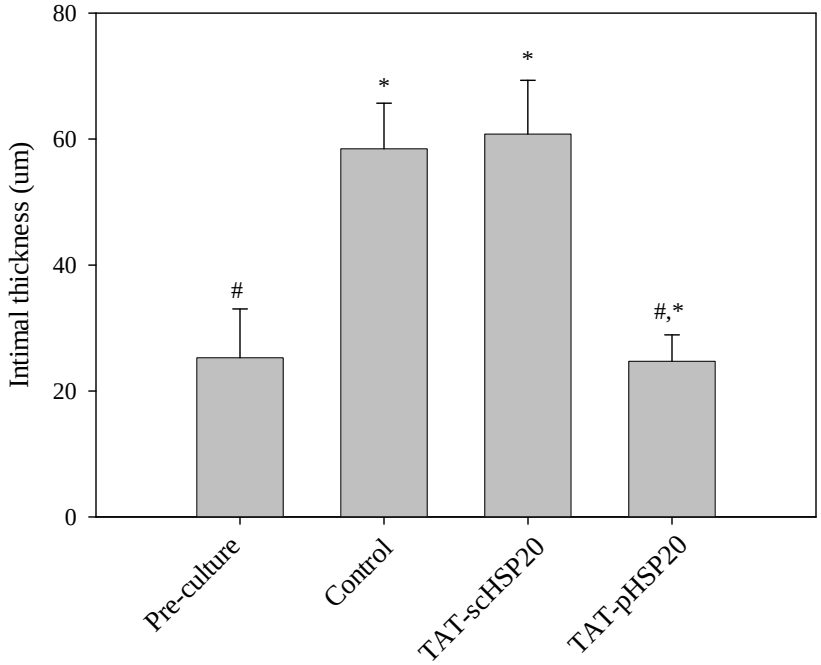

Figure 5. Six human saphenous veins were cultured for 14 days in media alone [(RPMI-1640, 30% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), L-glutamine (2 mmol/L)] or media containing peptides with the TAT transduction sequence linked to a scrambled amino acid sequence surrounding the HSP-20 phosphorylation site (TAT-scHSP20), or the TAT transduction sequence linked to the amino acid sequence surrounding the HSP20 phosphorylation site (TAT-pHSP20). Both of the HSP20 peptides contained a phosphoserine. Prior to culture several rings from each vein were fixed in formalin (pre-culture). TAT-pHSP20 maintained intimal thickness compared to pre-culture (# $P > 0.05$) and significantly inhibited intimal hyperplasia compared to the control and TAT-scHSP20 treated veins (* $P < 0.05$).

Intimal/medial ratios were determined morphometrically. There was no significant difference between TAT-pHSP20 treated veins and the pre-culture I/M ratio (0.08 vs 0.12 respectively; $P = 0.695$). In contrast, there was a significant increase in the I/M ratio between the pre-cultured veins and both the control (0.31; $P < 0.001$) and TAT-scHSP20 (0.36; $P < 0.001$) treated veins. There was no difference between the control and TATscHSP20 treated veins ($P = 0.24$). Additionally, there was a significant decrease in I/M ratio between TAT-pHSP20 treated veins and both the control ($P < 0.001$) and TATscHSP20 ($P < 0.001$) treated veins. These data suggest that phosphopeptide analogues of HSP20 inhibit intimal hyperplasia in human saphenous vein in an organ culture model.

Heparin can be used to control the release of TAT based peptides: Preliminary diffusion studies were performed by forming 10% 100 µl gels (2:1 heparin: PEG_{10}-$4HBD_1$) in the bottom of a 96-well plate (PEG_{10}-$4HBD_1$ refers to a 10,000 MW poly(ethylene glycol) four arm star polymer linked to 4 peptides of HBD1). Soluble PBD_2 or PBD_3, having 33.3 and 14 % relative affinity to heparin as compared to HBD1, was added to the gels (1:4 peptide:heparin) and allowed to incubate for one hour at room temperature. Control solutions consisted of PEG_{10}-$4HBD_1$ and PBD_3 without heparin. After incubation, the gels (n=6 of each type) were washed several times with PBS, pH 7.4 by adding 100 µl PBS and removing 100 µl of the solution. The extracted solution was examined spectrophotometrically for the presence of FITC (Abs 490 nm) label. Standard curves of PBD_2 and PBD_3 were used to calculate the fraction of each type of peptide removed during the wash steps.

Figure 6 shows the results of the preliminary diffusion studies. After 18 washes, all of the PBD_3 peptide in the control solutions had been recovered. The PBD_2 and PBD_3 peptides both had slower release profiles indicating that the heparin-based gels were effective in sequestering these peptides. After 18 washes, approximately 80% of the PBD_3 peptide had been recovered whereas only about 60% of the PBD_2 peptide was recovered. Different release profiles were expected since PBD_3 has a dissociation constant 1.8 times higher than that of PBD_2. These results indicate that the gels can release heparin-binding peptides at different rates based upon heparin affinity.

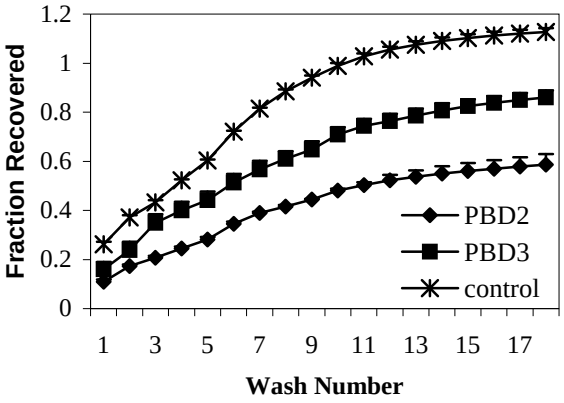

Figure 6. Physical PEG-co-Peptide and heparin gels containing PBD2 and PBD3 having 33 and 14 % relative affinity to heparin as compared to HBD1 in table 1 were placed in the bottoms of wells of 96 well plates and the gels were wash periodically over several hours. As a control, PBD2 was placed in wells without heparin and its release was observed.

D. Research Design and Methods

Aim 1. Optimize TAT-pHSP20 as a functionally active biomolecule

Develop and synthesize a panel of analogues of the TAT-pHSP20 peptides*:* The preliminary studies on vascular relaxation were performed using a 24 amino acid peptide: *YGRKKRRQRRR*GCWLRRAS*APLPGLK. The amino acids in italics are the TAT sequence, followed by a peptide sequence from the HSP20 molecule surrounding the phosphorylation site. This peptide was synthesized with a phosphoserine (S*). This peptide produced dose-dependent relaxation of porcine coronary artery that was maximal at a concentration of 1 mM. The relaxation was sustained for 30 minutes. A shorter peptide analogue, *RRRR**RR***AS*APLP, also produced relaxation of porcine coronary artery but at higher concentrations (10 mM). The full-length recombinant TAT-HSP20 induces relaxation in µM concentrations

(data not shown). This suggests that the full-length protein, with the complete tertiary structure is functionally active at lower concentrations than the peptide analogues. However, synthetic peptide analogues are easier to produce and purify, thus they represent an optimal pharmacologic moiety. Thus, we will optimize the peptide analogues so that they are functional at lower concentrations. We will focus on optimizing both the biomimetic analogue of HSP20 and the protein transduction domain. The transduction domain will later be altered to achieve optimal delivery rates see aim #2. It may not be possible to completely decouple delivery and function, but initially this will be done to limit the complexity of the problem.

Several groups have worked to create short, *de novo* peptides with stable secondary. With the exception of peptides that are coordinated with metal ions or disulfide bridges there are few examples of structure-stable peptides shorter than 35-50 amino acids (Neidigh et al. 2002). A recent example of a stable miniprotein, 20 amino acids in length, composed of only naturally occurring amino acids, is given by Neidigh et al. The designed peptide has a hydrophobic domain and an α-helical domain. *De Novo* design is generally begun with a desired secondary structure in mind, and steps are taken to stabilize that structure (De Grado and Summa 1999). Since the structure of the parent HSP20 molecule has not yet been determined and since it is difficult to obtain the structure of short linear conformations due to their ability to rapidly sample many configurations, the optimization of the pHSP20 peptide will be undertaken using a systematic approach recently reviewed by (Hruby 2002). This approach allows for the identification of crucial side chains, provides clues into the optimal secondary structure of the peptides, attempts to stabilize the stable secondary structure and later begins to investigate the importance of the side chain configurations and their dynamics. This study stops short of investigating side chain dynamics, but it importance is not underestimated and future studies will investigate these parameters.

As it is believed that the shortest functional peptides has been identified, the first steps in optimization will include alanine scans, d-amino acid scans and proline scans to determine the importance of the function of each individual amino acid with respect to interaction with the target molecule and to determine structure of the peptide. The alanine scan will help to determine importance of the amino acid side chains in interaction with the target while the d-amino acid (helps stabilize or destabilize secondary structure) and proline (helps stabilize β-turns) scans will help determine importance of an amino acid in determining secondary structure. These scans will be done by swapping out each individual amino acid in the sequence WLRRAS*APLPGLK for alanine, the complimentary d-amino acid and proline (Hruby 2002). Table II shows sequences to be made and evaluated.

TableII. Sequences to be synthesized and tested for functionality during optimization process.

Alanine Scan	**D-Amino Acid Scan**	**Proline Scan**
TAT- ALRRAS*APLPGLK	TAT- dWLRRAS*APLPGLK	TAT- PLRRAS*APLPGLK
TAT- WARRAS*APLPGLK	TAT- WdLRRAS*APLPGLK	TAT- WPRRAS*APLPGLK
TAT- WLARAS*APLPGLK	TAT- WLdRRAS*APLPGLK	TAT- WLPRAS*APLPGLK
TAT- WLRAAS*APLPGLK	TAT- WLRdRAS*APLPGLK	TAT- WLRPAS*APLPGLK
#	TAT- WLRRdAS*APLPGLK	TAT- WLRRPS*APLPGLK
TAT- WLRRAA*APLPGLK	**	TAT- WLRRAP*APLPGLK
#	TAT- WLRRAS*dAPLPGLK	TAT- WLRRAS*PPLPGLK
TAT- WLRRAS*AALPGLK	TAT- WLRRAS*AdPLPGLK	##
TAT- WLRRAS*APAPGLK	TAT- WLRRAS*APdLPGLK	TAT- WLRRAS*APPPGLK
TAT- WLRRAS*APLAGLK	TAT- WLRRAS*APLdPGLK	##
TAT- WLRRAS*APLPALK	TAT- WLRRAS*APLPdGLK	TAT- WLRRAS*APLPPLK
TAT- WLRRAS*APLPGAK	TAT- WLRRAS*APLPGdLK	TAT- WLRRAS*APLPGPK
TAT- WLRRAS*APLPGLA	TAT- WLRRAS*APLPGLdK	TAT- WLRRAS*APLPGLP

Residue is already an alanine; no substitution required at this position.
** Residue not available in d-form
Residue is already a proline; no substitution required at this position.

In addition, each amino acid will be synthesized on both rink amide resin and Wang resin to determine the importance of amine protected C-termini or free C-termini respectively. Peptides will also be synthesized with the final Fmoc left in place and with the final Fmoc cleaved to determine the importance of protected N-termini and free N-termini respectively.

Peptides will be synthesized on either rink amide resin or Wang resin using standard Fmoc chemistry on an Advanced ChemTech APEX peptide synthesizer in the PI's laboratory (Brodanzski 1993). Using a 96 well block, the APEX allows us to simultaneously synthesize up to 96 peptides on a 0.05 mmol scale. For those peptides that need to be synthesized on a larger scale, the 40-well block (up to 40 peptides on a 0.2 mmol scale) will be used. Following synthesis, the peptides will be cleaved from the resin with a trifluoroacetic acid based cocktail, precipitated in ether and recovered by centrifugation. The recovered peptides will be dried overnight *in vacuo*, resuspended in MilliQ purifed water and purified using and ÄKTA FPLC equipped with a C18 prep scale column. An acetonitrile gradient will be used to achieve purification. A small sample of peptide will be solubilized in 0.1% formic acid and an equal mixture of water and acetonitrile so that the peptide mass can be confirmed by electrospray mass spectrometry using a Waters' capillary HPLC and Micromass Quadrupole-time of flight-hybrid mass spectrometer.

***In Vitro* Characterization of Peptide Activity:** Each of the peptides initially will be screened for activity using an *in vitro* cell culture model. Activation of cyclic nucleotide signaling pathways in many cultured cells leads to dynamic alterations of cytoskeletal structure termed "stellation (Cechin et al. 2002)." We have recently determined that PTD-pHSP20 analogues also cause stellation (Figure 7). Thus, this provides a rapid simple assay of activity. Swiss 3T3 cells will be grown in 96 well plates, and incubated in the presence of the peptides (concentrations) for 1 hour. The cells will be fixed and stained with Alexafluor-conjugated phalloidin. Images will be taken on an inverted Leica DM IRBE microscope with fluorescence. This provides a qualitative assay of peptide activity. Peptides, which induce stellation in the cell culture model, will be assayed for activity in a muscle bath. To date, all peptide analogues, which have biologic activity in the muscle bath, also induce stellation. Since the muscle bath experiments require significant time to perform, we will use stellation as a model system to prescreen our peptides.

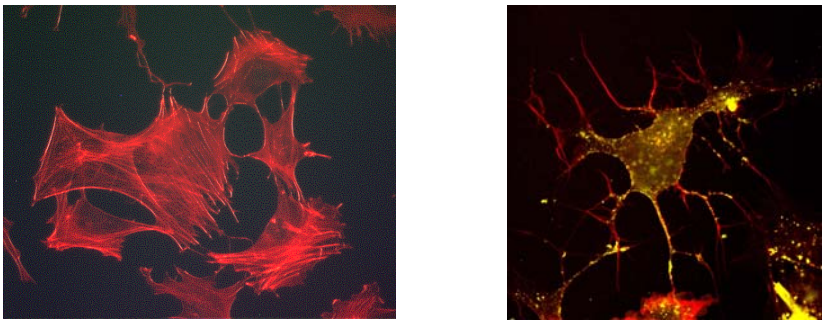

Figure 7: Fibroblasts were grown on glass slides for 24 hours. Media was exchanged for serum free media. **A.** Cells were maintained in serum free media for 1 hour then fixed with 4% paraformaldehyde and stained with Texas Red-phalloidin. B. cells were switched to serum free media containing 20 mM FITC-HSP20 for 1 hour then fixed with 4% paraformaldehyde and stained with Taxas Red-phalloidin. The untreated cells, A, remain polygonal while the treated cells adopt a stellite morphology in response to the TAT-pHSP20. The green staining in the nucleus is due to the FITC label. Controls were also done with a scrambled FITC-TAT-pHSP20; these controls remained polygonal.

Bioactivity of pHSP20 analogues *ex vivo*: The physiologic activity of the optimized TAT-pHSP20 analogues will be determined using segments of pig coronary arteries obtained from an abattoir. These tissues are obtained on a weekly basis and are inexpensive. Rings will be cut from the coronary arteries and placed in a muscle bath. The optimal length tension relationship will be determined with repeated contractions using high extracellular KCl. The strips will be washed in bicarbonate solution between contractions and allowed to equilibrate for at least 30 minutes.

To determine the vasorelaxing properties of the peptides, rings of porcine coronary artery will be pre-contracted with serotonin (10^{-6} M, a dose which just produces maximal, sustained contraction) and then treated with sequentially increasing doses (10^{-8} – 10^{-3}M) of the peptide analogues. Peptides that produce maximal relaxation at the lowest concentration then will be screened for duration of action. This provides a quantitative, reproducible bioassay of the engineered biomolecules.

Cyclic Peptide Derivatives: Once knowledge has been gained in the importance of amino acid side chains from alanine scans and insight into potential preferred secondary structures has been obtained from proline and D-amino acid scans, studies will be done on cyclic variants that are known to help stabilize secondary structure. It is crucial to know which amino acids are necessary for function prior to planning the cyclic structures. Care must be taken during cyclization chemistry not to make use of side chains required for function. Crucial side chains cannot be so constrained that they are unable to function in coordination with the acceptor molecule, yet must be restricted to a relative conformation that allows for optimal function. Several different chemistries have been developed and used for formation of cyclic peptides including: disulfide bonds, backbone-backbone, N-terminal-backbone-N-terminal-C-terminal and C-terminal-backbone linkages. Dr. _____ has extensive experience in this area and all work will be performed in close collaboration with secondary mentor, Dr. _____. Since it is difficult to plan cyclization experience with knowledge of the results of the scan experiments, no elaboration of experimental details are presented. Making use of x-ray diffraction and NMR spectroscopy, future studies by collaborators will help elucidate the optimal structure of the optimized peptide.

Anticipated results and potential pitfalls: These experiments will determine the magnitude and duration of action of the engineered pHSP20. The focus of these experiments is to determine the optimal biomolecule for complete and sustained relaxation. The initial peptide analogues of pHSP20 induce relaxation at concentrations of 1 mM. The optimized molecules may be effective in inducing relaxation at lower concentrations than the peptide analogues. In addition, it will be determined if the duration of action (relaxation) is longer with the optimized PTD-pHSP20 peptide.

Taken together, these assays allow for rapid screening and more thorough dose dependent analysis of biological activity of the peptides. Peptides that are either more or less effective at inducing relaxation are expected. This data will be useful in predicting structure and optimized peptide sequences. Information on the importance of each amino acid in function and structure and will allow us to predict the optimal structure of the peptide and additional changes that may enhance peptide activity including cyclization or multiple amino acid substitutions. From initial observation of the parent pHSP20 peptide sequence, it is expected that alterations that stabilize β-turns will enhance peptide function; proline substitutions are most likely to have this stabilizing effect. If none of the peptide derivatives show improved function, it may be necessary to evaluate longer sequences from the full length HSP20 molecule or use of recombinant full-length PTD-HSP20.

We have obtained the full-length cDNA for the human HSP20 molecule from _____ (University of _____). Constructs have been engineered with the TAT sequence at the c-terminus of the HSP20 cDNA. These constructs were cloned into the pET14b vector and transformed into the BL21(DE2)pLysS strain of *E. co*li. High cell density fermentation (_____ et al. 1997) followed by protein purification using nickel affinity chromatography yielded functional $(His)_6$TAT-HSP20. The $(His)_6$TAT-HSP20 protein was determined to be functional in muscle bath studies at concentrations of 100 μM (data not shown).

Lessons Learned: At completion of specific aim 1, the PI will be proficient in *in vitro* cell culture, immunohistochemistry, *ex vivo* vascular tissue function evaluation and in peptide optimization techniques. The PI will be able to apply these techniques to address additional problems in vascular physiology and other areas of biomedicine. In addition, the PI will be proficient in the design and optimization of peptide therapeutics and will be able to apply the learned techniques to the discovery and development of therapeutics for other diseases.

Aim 2: Develop and characterize controlled release systems for TAT-pHSP20 to ensure sustained delivery of the biomolecule in an effective therapeutic concentration.

Dextran-co-polysaccharide gels will be formed based on affinity of heparin-binding peptides for heparin. Dextran will be functionalized with methacrylate groups to which heparin-binding peptides (HBD) will be coupled. Heparin will be added to the peptide-functionalized dextran on a 1:2 heparin:peptide ratio to form physical gels from which heparin-binding peptide therapeutics can be released (Figure 8).

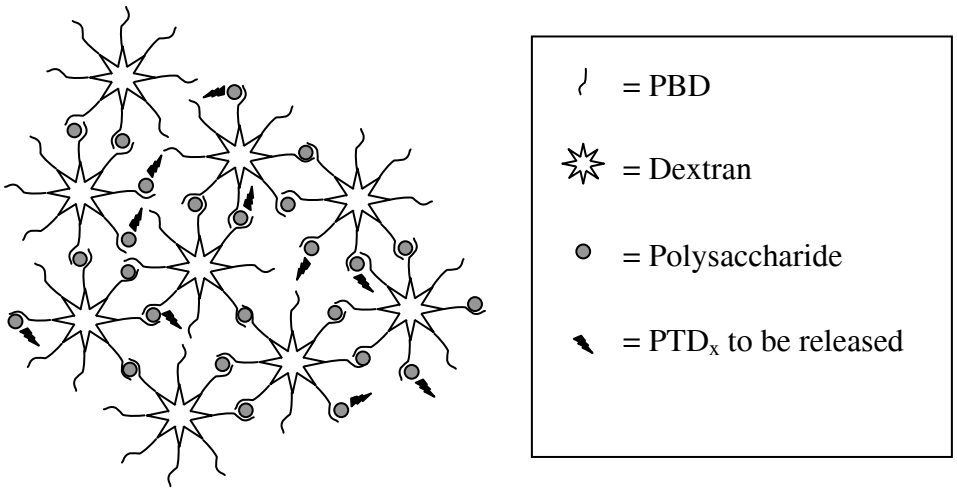

Figure 8. A cartoon representation of the physical gels formed when combining the functionalized dextran molecules with polysaccharides. Therapeutic peptides conjugated to protein transduction domains (PTD) can be added to the gel and then released at a rate dictated by the affinity of the PTD for heparin or other polysaccharide.

Peptide Synthesis: Polysaccharide binding peptides, shown in Table III, will be synthesized on rink amide resin using standard Fmoc chemistry on an Advanced ChemTech APEX peptide synthesizer in the PI's laboratory. Using an 8 well block, the APEX allows us to simultaneously synthesize up to 8 peptides on a 0.7 mmol scale or 1 peptide on a 5.6 mmol scale. In order to study the degradation rate of the gels, the peptides will be synthesized with dansyl-glycine on the amino-termini. Following synthesis, the peptides will be cleaved from the resin with a trifluoroacetic acid based cocktail, precipitated in ether and recovered by centrifugation. The recovered peptides are dried overnight *in vacuo*, resuspended in MilliQ purifed water and purified using and ÄKTA FPLC equipped with a C18 prep scale column. An acetonitrile gradient (from 10-60% acetonitrile) will be used to achieve purification.

Table III. Polysaccharide-binding peptides released from the gels.

Name	Sequence	K_D (M)	Reference
HBD‡	FITC-A(β)*KAFAKLAARLYRKA*GC	5.1±0.2 x 10^{-8}	(Tyler-Cross et al. 1994)
PTD_2‡	FITC-A(β)*AAFAKLAARLYRKA*GC	3.7±0.6 x 10^{-7}	(Tyler-Cross et al. 1994)
PTD_3‡	FITC-A(β)*KAFAALAARLYRKA*GC	6.6±0.1 x 10^{-7}	(Tyler-Cross et al. 1994)
TAT (PTD_4)	FITC-A(β)*YGRKKRRQRRR*	3.0±1.4 x 10^{-8}	(Rusnati et al. 1999)
PTD_5[a]	FITC-A(β)*YARKARRQARR*	To be determined	(Ho et al. 2001)

[a] This PTD has 33X the activity of TAT with significantly decreased heparin affinity. ‡ Each of the heparin-binding peptides was based on the heparin-binding domain from human antithrombin III. The dissociation constants correspond to the italicized domains.

Methacroylation of Dextran: Methacroylated dextran will be synthesized using methods described by van Dijk-Wolthuis et al (Dijk-Wolthuis et al. 1997). Dextran (MW 70kD) and dimethylaminopyridinine

(DMAP) will be dissolved in dimethylsulfoxide (DMSO) under nitrogen atmosphere at room temperature. Glycidyl methacrylate (GMA) will be added to the mixture to produce GMA-derivatized dextran (dex-GMA). The amount of GMA will be adjusted to obtain a degree of substitution (DS: molar ratio of GMA per glucopyranose residue) of 4%. The reaction will be terminated after 48 hours. The product then will be purified from the reaction mixture by solvent removal and size exclusion chromatography. Aqueous solutions of methacroylated dextran will be rapidly frozen in liquid nitrogen, lyophilized, and stored frozen. The average degree of substitution in purified product will be determined by analysis of NMR spectra.

Coupling peptide to Dextran via Michael Addition: Methacroylated dextran will be dissolved in PBS containing 2 mM ETDA, pH 7.4, at concentrations of 20 % (w/v). Peptide, HBD, will be dissolved in a second tube at molar concentrations equivalent to 5:1 moles of peptide or 10:1 moles of peptide:Methacroylated dextran; the pH then will be adjusted to 7.4 if necessary. (Ratio of peptide to dextran will be changed if it is determined during characterization that the gels are either too weak or too strong; the gels need to have storage moduli on the order of 1-5 kPa in order to spread appropriately on vascular tissue as determined in previous studies with PEG gels). The appropriate peptide and Methacroylated dextran solutions will be combined and allowed to react via Michael-type addition for 12 hours at 37°C. Previous studies indicate quantitative coupling of PEG-VS with thiol-terminated peptide to the limits of fluorescent detection methods (results not shown). HBD-conjugated dextran solutions then will be dialyzed against MilliQ water in 25,000 MWCO dialysis tubing (Spectrum) to remove possible residual uncoupled peptide and to exchange solvent. The resulting conjugated dextran solutions will be lyophilized and stored at –20°C.

Gelation: HBD-conjugated dextran will be dissolved in PBS to concentrations varying from 5-20 percent (w/v). In a separate tube, 18,000 MW heparin or 15,000 MW DS will be dissolved such that the molarity of the polysaccharide solution results in one polysaccharide molecule for every 2 peptide molecules attached to dextran in an equivalent volume. Combining 50 µl of both the HBD-conjugated dextran solution and the polysaccharide solution will make gels. Gel formation will proceed for 15 minutes, after which time; the gels will be centrifuged and evaluated for phase separation. Preliminary studies with conjugated PEG star polymers indicate that some shrinkage occurs upon gel formation when 10 percent gels are made with PEG_{10}-4HBD and heparin. We will attempt to eliminate shrinkage by optimizing polymer concentration. We also may need to vary the ratio of polysaccharide: HBD-conjugated dextran from 2:1 in order to eliminate or minimize shrinkage of the gels.

Mechanical Testing: Mechanical properties will be studied using a TA Instruments AR1000 Rheometer. Gels with a thickness of 1 mm will be loaded between two parallel plates. The upper plate will be driven in an oscillatory motion to achieve sinusoidal deformation of the sample. Frequency sweeps will be run to determine the linear viscoelastic region. Then, frequencies within the linear range will be studied, and the storage and loss modulus will be recorded (Tung and Dynes 1982; Chambon and Winter 1985). Mechanical testing data not only will indicate gel formation, but also will provide information on how the number of peptides conjugated to each dextran molecule affects G' and G''. Finally, the effect of heparin and DS will be evaluated.

Anticipated Results and Potential Pitfalls: Gels with larger ratio of peptide:dextran are expected to have higher G' as are gels made with heparin, 18,000 MW as compared to those made with dextran sulfate, 15,000 MW. Since the dextran sulfate molecules are, on average, shorter than the heparin molecules, the number of peptide interactions/molecule will be lower for dextran sulfate resulting in a lower G' value. Gels will first be optimized with respect to G'. As determined in previous studies on PEG based gels, a G' on the order of 1-5 kPa will have an optimal spreading characteristic for applying to vessels. We anticipate that the proposed gels will have the appropriate properties; however, if properties are not ideal, further experiments will be done to improve the properties. Optimization can be done in two ways; the first is to alter the overall polymer concentration in solution. The second is to alter the crosslink density of the gel, which will be done by varying the number of peptide molecules conjugated to a dextran molecule. Both approaches will be used to optimize the gel if necessary.

Model to Predict the Release Rate: In order to model the release of peptides from the gels, a mass balance must be written for each diffusible species within the gel. The diffusible species include: HBD-conjugated dextran, (V), polysaccharide (H) and protein-transduction peptide (P) (Table III). Equations 1-4 indicate the states in which these species can exist and show the on and off rate constants that govern the states. For example, equation 1 shows that P can exist unbound or bound to H; however, the state of P is also coupled to equation 3, which shows that P can also exist bound to V and H. In all, six states can exist. These

equations are written assuming that binding of one peptide to polysaccharide does not influence the binding of additional peptides to the same polysaccharide molecule.

$$H + P \underset{k_1^-}{\overset{k_1^+}{\rightleftharpoons}} HP \qquad (1)$$

$$V + H \underset{k_2^-}{\overset{k_2^+}{\rightleftharpoons}} VH \qquad (2)$$

$$VH + P \underset{k_3^-}{\overset{k_3^+}{\rightleftharpoons}} VHP \qquad (3)$$

$$V + HP \underset{k_4^-}{\overset{k_4^+}{\rightleftharpoons}} VHP \qquad (4)$$

The K_D is known for all but one of the proposed peptides (Tyler-Cross et al. 1994; Rusanti et al. 1997). Because $K_D=k_{off}/k_{on}$ we need only to determine k_{off} for all but the modified TAT peptide.

The experimental release study geometry most closely resembles that of a sphere. For comparison, the model will be solved for a spherical geometry. Mass balances are first written for each species assuming that each species is diffusible. Diffusion coefficients and on and off rates of each species are required for the mass balances. Equations 5-10 are the 6 mass balances required for the six species shown in equations 1-4.

$$\frac{\partial c_P}{\partial t} = D_P \nabla^2 c_P - k_1^+ c_H c_P - k_3^+ c_{VH} c_P + k_1^- c_{HP} + k_3^- c_{VHP} \qquad (5)$$

$$\frac{\partial c_V}{\partial t} = D_V \nabla^2 c_V - k_2^+ c_V c_H - k_4^+ c_V c_{HP} + k_2^- c_{VH} + k_4^- c_{VHP} \qquad (6)$$

$$\frac{\partial c_H}{\partial t} = D_H \nabla^2 c_H - k_1^+ c_H c_P - k_2^+ c_V c_H + k_1^- c_{HP} + k_2^- c_{VH} \qquad (7)$$

$$\frac{\partial c_{HP}}{\partial t} = D_{HP} \nabla^2 c_{HP} + k_1^+ c_H c_P + k_4^- c_{VHP} - k_1^- c_{HP} - k_4^+ c_V c_{HP} \qquad (8)$$

$$\frac{\partial c_{VH}}{\partial t} = D_{VH} \nabla^2 c_{VH} + k_2^+ c_V c_H + k_3^- c_{VHP} - k_2^- c_{VH} - k_3^+ c_{VH} c_P \qquad (9)$$

$$\frac{\partial c_{VHP}}{\partial t} = D_{VHP} \nabla^2 c_{VHP} + k_3^+ c_{VH} c_P + k_4^+ c_V c_{HP} - k_3^- c_{VHP} - k_4^- c_{VHP} \qquad (10)$$

It is assumed that initially all species are in equilibrium, and the spheres are 100 µl in volume. We are also setting boundary conditions at the interface of the sphere and the solution to be reflective. This will help to reduce artifacts that occur when conditions are artificially set to zero. Equations 11-18 govern the initial condition that all species are in equilibrium.

$$c_H^{eq} c_P^{eq} k_1^+ = c_{HP}^{eq} k_1^- \qquad (11)$$

$$c_V^{eq} c_H^{eq} k_2^+ = c_{VH}^{eq} k_2^- \qquad (12)$$

$$c_{VH}^{eq} c_P^{eq} k_3^+ = c_{VHP}^{eq} k_3^- \qquad (13)$$

$$c_V^{eq} c_{HP}^{eq} k_4^+ = c_{VHP}^{eq} k_4^- \qquad (14)$$

$$c_P^{total} = c_P^{eq} + c_{HP}^{eq} + c_{VHP}^{eq} \qquad (15)$$

$$c_H^{total} = c_H^{eq} + c_{HP}^{eq} + c_{VH}^{eq} + c_{VHP}^{eq} \qquad (16)$$

$$c_V^{total} = c_V^{eq} + c_{VH}^{eq} + c_{VHP}^{eq} \qquad (17)$$

$$c_i = c_i^{eq}, \; for \; i = P, H, V, HP, VH, VHP \qquad (18)$$

Equations 11-17 are coupled nonlinear equations, which can be solved separately for the equilibrium

concentrations. The known values can be input into equations 5-10 along with the k_{on} and k_{off} values and diffusion coefficients for t=0 at r ≥ 0. The nonlinear set of equations represented by equations 5-8 then will be solved numerically using MatLab. Values obtained from this model will be compared with experimental values.

Determination of kinetic parameters: In order to solve the defined model, the eight on and off rates and the diffusion coefficients for each species must be known. Rates will be determined using a BIAcore 1000 (Pharmacia Biosensor AB). Peptide will be immobilized to the sensor surface using the C-terminal cysteine thiol. The sensors can be purchased from Pharmacia Biocore AB and come with a carboxymethylated dextran surface, which can be derivatized to allow for thiol coupling with the peptide. To derivatize the surface for coupling with thiols, 10 µl of 100 mM N-hydroxysuccinimide and 400 mM EDC are injected. Next, 15 µl of 40 mM cystamine dihydrochloride in 0.1 M borate buffer (pH 8.5) and 15 µL of 100 mM 1,4-dithiotherythritol in 0.1 M borate buffer (pH 8.5) are injected. This readies the surface for coupling to the thiol of the peptide. Peptide, 100 µg/ml, dissolved in 10 mM sodium citrate buffer will be injected into the sensor in a volume of 20 µl. Unreacted surface functional groups will be blocked by addition of 20 µl 2-(2-pyridinyldithio)ethaneamine (PDEA), 20 mM, 1 M NaCl in 0.1 M formate buffer (pH 4.3). In order to normalize the amount of peptide conjugated on different chips, the amount of immobilize peptide will be determined in RU which corresponds to the initial BIAcore baseline prior to immobilization and the baseline after immobilization (Zeng et al. 1998).

Analysis of the on and off rates for heparin and the immobilized peptide will be determined by injecting a heparin solution at concentrations of 0.1X K_D, 1.0X K_D and 10X K_D. The heparin will be allowed to bind until a steady RU is reached. Then, the solution will be replaced with HEPES buffered saline, pH 8.0 (HBS). To evaluate the association and dissociation rates, the BIAcore measures changes in surface plasmon resonance response at 25°C over time; the BIAevaluation software will use the RU data to calculate kinetic parameters. Regeneration of the biosensor surface can be achieved by adding 100 mM HCl followed by washing with (HBS). Between 2-4 chips will be used for the evaluation of each peptide; three replicates of both k_{on} and k_{off} will be measured on each chip. (Zeng et al. 1998; Choulier et al. 1999).

Determination of Diffusion Coefficients: Diffusion coefficients will be measured using dynamic light scattering (Bell et al. 1997). All measurements will be done using a Protein Solutions Dyna Pro Molecular Sizing Instrument MS/X. The Dynamics V6 software package will be use for all data sets. Solutions of peptide, HBD-conjugated dextran, polysaccharide or a combination of these molecules will be transferred into the 12 µl sample compartment and illuminated by a 50 mW, 824.8 nm solid-state laser. Solutions on the order of 1-10 mg/ml will be used. The intensity of the light scattered at 90° will be measured. The instrument and software measure a radius of hydration R_H, which can be used in turn to calculate the diffusion coefficient using the Stokes-Einstein equation:

$$D_T = k_b T/6\pi\eta R_H \qquad (19)$$

Where k_b is Boltzmann's constant, T is absolute temperature and η is the solvent viscosity. The measured diffusion coefficients will be used to solve the proposed model.

Anticipated results and potential pitfalls*:* We are assuming that hydrogels that are on the order of 90% water will not impede diffusion of the therapeutic to be released. Therefore, the measured diffusion coefficients are in solution only and may not reflect the values of the diffusion coefficients of the species within the proposed gels. Initially, these values will be used in the model. However, if model predictions do not match experimental results, diffusion will be reevaluated in gels using light microscopy by incorporating fluorescently tagged molecules into the center of the gels. The gels will be incubated for varying periods of times, and images will be taken of the gels at these time points. The distance the labeled molecules travel will be evaluated by comparing the location of the new dye-front with the original front. Analysis of the distance traveled within a defined time interval will allow us to approximate a diffusion coefficient for each fluorescently tagged molecule. If models still do not accurately predict experimental release from the gels, both k_{on} and k_{off} will be determined using the Biocore instead of relying on published K_D data and measured k_{off} data to calculate k_{on}. If the proposed model is still unable to predict experimental results, Dr._____ and I will work to refine the model.

Once an appropriate model has been defined for heparin, the model will be evaluated for DS. It will be necessary to determine diffusion coefficient for DS and to determine on and off rates for DS peptide pairs.

Characterize release of bioactive peptide: Gels will be made as described above with the exception that the FITC-coupled therapeutic to be released will be added to the gel formulation prior to gelation. Gels, 100 µl in volume, will be made in the bottoms of 15 ml tubes. Only those gels that have been optimized to eliminate shrinkage and to have suitable mechanical properties will be evaluated. At time intervals of 1, 2, 4, 8, 24 and 48 hrs and every other day until release is complete, 1 ml aliquots will be removed and the volume will be replaced with 1 ml of PBS, pH 7.4. All aliquots will be evaluated for FITC-labeled peptide, dansyl-labeled HBD-conjugated dextran and Texas Red-labeled heparin release using a Molecular Devices Gemini EM microplate spectrofluorometer. If necessary, other time points will be evaluated as deemed necessary after initial experiments. DS and heparin containing gels will be compared to one another to evaluate release as will release of different PTD-conjugated therapeutic peptides be compared to one another. Target release times from 4 hours to 2 weeks will be targeted for shaphenous vein bypass as 4 hours is sufficient to complete bypass surgery and work by West et al. has demonstrated that short-term inhibition of smooth muscle cell activation can inhibit long-term intimal hyperplasia (West and Hubbell 1996). Extended release times will allow comparison of implants treated only for 4 hours verses those that have been treated for up to two weeks. *Six replicates of each gel type will be studied and ANOVA with $p \leq 0.05$ will be used to determine significance in the differences of release between different groups.*

The release rates determined experimentally then will be compared with the values determined theoretically. This comparison provides an iterative step that will allow us to optimize the model if significant variations exist. A good mathematical model will allow us to predetermine the required polysaccharide binding affinity of molecules and required concentration and ratios of constituents in order to achieve the desired release rate.

Anticipated results and potential pitfalls: These experiments will evaluate the mechanical properties of the gels. The physical gels may not be stable enough for the controlled release, or may not gel at all. If either of these occurs, synthesizing the peptides with cysteine residues on either end will make covalently crosslinked gels. The ester bond formed during conjugation of the peptide to dextran may not be stable enough to maintain gel integrity for controlled release. If this occurs, the peptides will be synthesized with protecting groups on lysine and arginine groups that are not removed during resin appropriate cleavage conditions. Then the primary amine group on the peptide will be conjugated to aldehyde functionalized dextran and the lysine and arginine protecting groups will be remove after conjugation is complete.

Optimized HSP20 peptides will improve the commercial potential of HSP20 as a therapeutic for vascular bypass surgery. The technology to synthesized optimized P20 peptides will be transferred to AzERx, LLC. The preliminary data for this proposal was obtained through research funded by a phase 1 STTR grant to AzERx, LLC (_____).

E. Human Subjects
N/A

F. Vertebrate Animals
N/A

G. Literature Cited
[Redacted]

H. Consortium/Contractual Arrangements
N/A

I. Consultants
N/A

7. Checklist

8. Appendix