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.**

The PI performs research in the area of vascular biology with emphasis on the role of fluid forces in regulating cell and biomolecule function. _____ laboratory develops and applies quantitative experimental methods to complement traditional methods in molecular biology and hematology, in order to gain novel insight into the pathways regulating leukocyte-endothelial cell adhesion and thrombosis mechanics. In the long run, we aim to apply this understanding of biological mechanisms and fluid-flow mediated phenomena to develop new drugs and therapeutic strategies designed to treat vascular diseases. Some aspects of this work are funded by the _____.

In recent experiments, we applied our quantitative experimental strategies to study shear-induced platelet activation. Here, we observed using static and dynamic light scattering, that fluid shear in addition to regulating cell function may also regulate bio-molecular structure. In this study, fluid forces induced the self-association of plasma protein von Willebrand Factor (vWF). We study this observation in greater detail in the current proposal. The objective of the training component of this proposal is to obtain formal training in methods that can be used to study biomolecule structure and function under shear. These methods include light scattering, small-angle X-ray and neutron scattering, surface plasmon resonance and molecular simulations. Some aspects of the training require the PI to attend graduate-level courses and workshops in areas including Research Ethics. Other aspects of training are unstructured and they involve self-study by the PI and interactions with collaborators. The specific research goals of the project are: i) to study the dynamic structure of plasma vWF under shear using light, neutron and X-ray scattering. ii) to quantify the rate and shear-dependence of vWF self-association in a milieu that mimics in vivo conditions using surface plasmon resonance. iii) to determine the structural features of biomolecules that make them susceptible to conformational alteration upon application of physiological and pathological fluid forces. This is achieved by combining the experimental knowledge obtained from the above studies with theoretical methods in molecular simulations and other computational techniques. In the long run, the studies aim to provide new mechanistic insight and therapeutic strategies to counter cardiovascular diseases.

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

Bioengineering Laboratory, _____ Dept., _____

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
_____	Assistant professor, _____	Principal Investigator
_____	Professor, _____	Department Head
_____	Associate Professor, _____	Consultant
_____	Assistant Professor, _____	Consultant
_____	Senior Scientist, _____	Consultant

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

Principal Investigator/Program Director (Last, first, middle):

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
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(Substitute Page)

Page Numbers

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4. Budget for Entire Proposed Period of Support (Form page 5) .....

5. Biographical Sketches *(Candidate and Sponsor[s]*—Biographical Sketch Format page) (Not to exceed four pages)*

6. Other Support Pages for the Mentor (not the candidate) .....

7. Resources (Resources Format page) .....

Section II: Specialized Information

1. Introduction to Revised Application (Not to exceed 3 pages) .....

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3. The Candidate

A. Candidate's Background .....

B. Career Goals and Objectives: Scientific Biography ..... *(Items A-C included in 25 page limit)*.....

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4. Statements by Sponsor(s), Consultant(s)*, and Collaborator(s)*

5. Environment and Institutional Commitment to Candidate

A. Description of Institutional Environment.....

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A. Statement of Hypothesis and Specific Aims .....

B. Background, Significance, and Rationale ..... *(Items A-D included in 25 page limit)* .....

C. Preliminary Studies and Any Results .....

D. Research Design and Methods .....

E. Human Subjects* .....

List appropriate grants with IRB approval dates or exemption designation .....

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List appropriate grants with IACUC approval dates or exemption designation .....

G. Literature Cited .....

H. Consortium/Contractual Arrangements*.....

I. Consultants*.....

7. Checklist

8. Appendix *(Five collated sets. No page numbering necessary)* ☒ Check if Appendix is included

Number of publications and manuscripts accepted for publication *(not to exceed 6)* 5

List of Key Items:

_____

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

Laboratory: The Bioengineering laboratories located within the Department of _____at _____is a 6,000 sq. ft. facility dedicated to the study of cellular bioengineering. The core facility comprises of 3 tissue culture rooms, 3 environmental rooms, a central autoclave facility and a media preparation room. The tissue culture facilities is equipped with several CO_2 incubators, laminar flow hoods, liquid nitrogen cell banks, -20°C and -80°C deep freeze refrigerators, Dupont-Sorvall centrifuges (RC80, RC-5B, RT-6000, RTlegend), a bench top microscope (Nikon), extensive glassware, pipettes, etc. Surrounding these core facilities, are the bioreactor laboratory, analytical measurement facilities and cellular biomechanics laboratory.

All of these facilities are readily available to the PI.

Clinical: None

Animal: None

Computer: The computational and word processing work outlined in the project will be performed on an IBM Pentium available in the PI's office. Several IBM PCs, Macintoshs and an SGI Indigo machine are also available in the PI's Laboratory to accomplish the computational aspects of this project. We also regularly use the ____ supercomputing facility for more rigorous calculations outlined in the proposal.

Office: The PI's 600 sq. ft. office space is located adjacent to the _____ Laboratory.

Other: The PI has access to specialized equipment in the_____ lab (see enclosed letter) for light scattering experiments. As outlined in the letter of collaboration, a rheometrics viscometer in this lab may also be used for rheo-optics measurements.

A Biacore-X instrument is available in the shared-user facility of the university. The PI has ready access to this since the School of _____ paid _____ for the acquisition of the instrument.

MAJOR EQUIPMENT:

Central for the proposed project are the cellular biomechanics laboratory and the analytical measurement facilities in the PI's Bioengineering lab. The cellular biomechanics laboratory is equipped with a Leitz Artisoplan microscope and Nikon Diaphot inverted microscope. There is also a CK40 (Olympus) inverted light microscope enclosed in a custom built temperature control incubator for the flow chamber experiments. These microscopes are attached to a time lapse video recorder via CCD camera (MTI, Inc., CCD-72), cooled CCD camera (Retiga-1300C, Qimaging) and a Kodak digital camera. Also available is a PC-based imaging system for video microscopy and digital image processing. There are also two viscometers (manufactured by Brookfield and Haake) that are used to study cellular adhesion in suspension. Analytical measurement facilities available include a FACS Calibur flow cytometer, HPLC workstation (HP-1090) with fluorescence detector (P-1046), Pharmacia FPLC, Flow Labs 96-well microplate reader (MK-22), Molecular Devices Fluorimeter, Coulter Counter (ZM). Molecular biology facilities for both proteins and nucleic acids work is also available including gel documentation facility, dark room for chemiluminescence, Biorad (Qcycler) PCR machine and a Beckman scintillation counter. Radioactivity work is approved in the laboratory.

Section II: Specialized Information

1. Introduction to Revised Application

2. Letters of Reference

3. The Candidate

A. Candidate's Background

The candidate graduated with a Ph.D. in Chemical/Biomedical Engineering from _____ in 1996, and conducted post-doctoral research in the Section of _____ (_____Dept.), _____ from 1995-97. During these years, the PI interfaced with numerous biologists and medical scientists at the _____ on projects related to blood and vascular disorders. Many of these interactions continue today with previous collaborators providing valuable, unique reagents for the PI's laboratory in addition to stimulating scientific discussions.

The focus of the work during the Ph.D. and postdoctoral research was on the mechanism of leukocyte binding to the vascular endothelium *in vitro* in models of inflammation. During these projects we:

1) Developed a series of experimental systems and mathematical analysis methodologies to quantitatively study leukocyte adhesion and motility mechanics in the presence (1) and absence of fluid shear (2, 3).
2) Performed experiments with human neutrophils (1, 4, 5) and lymphocytes (2, 6, 7) to examine cell binding mechanics via adhesion molecules belonging to the selectin, β_1-integrin[1] and β_2-integin families.
3) Demonstrated that below a maximal level of activation, selectin ligation and chemotactic activation act in synergy to amplify neutrophil activation, adhesion and transmigration (8). These features together control the rate at which leukocytes transition from rolling to firm-arrest.
4) Showed that at low shear rates, the contact time for cell-cell interaction in suspension may be high enough to allow neutrophil mediated firm arrest via a selectin independent pathway (9). In this paper, we also showed that L-selectin mediated cell adhesion is optimum only above a low/minimal/threshold shear rate.
5) Finally, during this time, in experiments that examined β_2-integin function under shear we illustrated that LFA-1 (Leukocyte Function Antigen-1) affinity for ICAM-1 (Inter-Cellular Adhesion Molecule-1) is greater than the avidity of Mac-1 (Macrophage antigen-1) interaction with ICAM-1. Further, while human blood neutrophil adhesivity was rapidly augmented within seconds after chemotactic stimulation, leukocyte adhesion via the β_2-integin subunits LFA-1 and Mac-1 decreases subsequently, over a period of minutes (10). This decrease in cellular adhesivity may regulate the transition of cells from firm-arrest to initiate transmigration.

In Fall of 1997, the PI moved to _____ as a tenure-track Assistant Professor of _____, and initiated independent research work. Currently, there are 6 Ph.D. students and 1 M.S. student in the PI's group. **Broadly, the projects in our laboratory focus on three areas**:

- Examination of the aspects of fluid shear regulating neutrophil adhesion function, platelet activation and protein morphology. Aspects of this work are the subject of the current proposal.

[1] Abbreviations: LFA-1: Leukocyte function antigen-1; Mac-1: Macrophage antigen-1; ICAM-1: Intercellular adhesion molecule-1; ICAM-3: Intercellular adhesion molecule-3; β_1-integrin=CD29 antigen; β_2-integin=CD18 antigen; vWF: von Willebrand Factor; vWD: von Willebrad Disease; GpIb: Glycoprotein-Ib (CD42); SANS: small-angle neutron scattering; SAXS: small-angle X-ray scattering; SPR: surface plasmon resonance; NCNR: NIST Center for Neutron Research; SEAS: School of Engineering and Applied Sciences

- Studies on selectin-ligand function with special emphasis on core-2 glycans. In this study, we are attempting to develop antagonists directed against selectin binding based on O-glycan structure of its ligand.
- Application of systems biology to examine the time-dependent changes in neutrophil function after stimulation.

In the research projects conducted at _____ we have shown that:

1) Inter-Cellular Adhesion Molecule-3 (ICAM-3) can bind LFA-1 on neutrophils to mediate neutrophil homotypic adhesion. We also expanded on our observations of the role of fluid shear and time in regulating β_2-integrin function (11), by demonstrating that while both β_2-integrin subunits, LFA-1 and Mac-1, contribute equally to neutrophil adhesion initially, their relative contributions to leukocyte adhesion differ markedly over the time course of stimulation. Adhesion via LFA-1 decreases four times faster than that via Mac-1. Perhaps, for this reason, while LFA-1 plays a more prominent role during cell adhesion to the vascular endothelium Mac-1 engagement is more critical for leukocyte transmigration.
2) Further, we have developed novel data analysis strategies in the parallel-plate flow chamber device, which is used to study leukocyte and platelet adhesion under shear (12, 13). We have also published data on the on-rates of selectin bonds both in suspension (14) and on substrates (15). While much attention in literature has been devoted to quantifying the off-rates of bio-molecules, these represent one of the first studies to quantify selectin on-rates under shear.
3) More recently, we have observed that fluid shear forces can act in synergy with chemotactic stimulus to upregulate neutrophil Mac-1 expression and affinity (manuscript in preparation). These hydrodynamic forces may be an added parameter regulating the transition from selectin mediated rolling to integrin mediated firm arrest.
4) We have also conducted extensive studied on the binding of selectins to core-2 based oligosaccharide ligands and the role of 3-O-Gal:sulfotransferases in formation of selectin-ligands in tumor cells (manuscripts in preparation).

Many aspects of the work discussed above are funded by the PI's grant from _____. A brief description of the specific aims of this grant follows on the next page.

The research environment at _____ and other interests in our laboratory have prompted us to extend our work funded as part of the _____ grant towards the study of fluid forces in controlling thrombosis: specifically the studies focus on the interplay between fluid shear, platelet-surface receptor GpIb (Glycoprotein-Ib) and plasma protein von Willebrand factor (vWF) in regulating platelet function. This is the focus of the current proposal. In a paper published from our laboratory earlier this year in _____, we present some of our findings:

- Here, we provided definitive proof of the role of fluid shear stress rather than shear rate in controlling shear-induced platelet activation. We also demonstrated that linear fluid shear alone, in the absence of non-linear flow, can regulate platelet activation. Further, for the first time, we estimated/quantitated the magnitude of fluid or hydrodynamic forces[2] applied on GpIb and vWF during cell activation.
- In a significant new finding, we also proposed a two-step mechanism for platelet activation where the binding of plasma vWF to platelet GpIb under shear is separable from the subsequent step of platelet activation.
- Finally, we demonstrate for the first time that fluid shear forces can induce the aggregation or self-association of plasma von Willebrand Factor (vWF). Previous studies have shown that the binding of

[2] Hydrodynamic forces refers to forces applied on cells and molecules due to the motion of fluid (e.g. blood flow).

vWF to platelet GpIb is augmented by fluid shear (17). The current studies thus demonstrate a novel effect of fluid flow on vWF structure.

_____

To better understand the effect of fluid forces on cells and biomolecules, we have also developed some careful and rigorous mathematical schemes to quantify fluid forces and its effect on cell adhesion and protein structure under physiologically relevant conditions (18, 19). Finally, to extend the experimental methodologies in our laboratory, we have undertaken studies, which employ dynamic and static light scattering and small-angle neutron scattering (SANS). The last studies (SANS) have been carried out through beam-time allocated by the _____. These studies have revealed that plasma vWF exists in solution in an elongated form and provide proof-of-principle that SANS is a reasonable approach to assess certain aspects of the vWF shape/morphology (see Prelim. Data section).

B. Career Goals and Objectives: How the award fits into past and future work

All the projects in our laboratory focus on inflammatory and thrombotic disorders. **The overall objectives are**:

- To develop and apply novel engineering tools to problems in medicine, with the objective of gaining a fundamental understanding of the mechanisms regulating human vascular pathologies. The application of bioengineering methods allows us to address problems in this field in a manner distinct from typical biologists and medical scientists.
- To use our understanding of biological mechanisms and fluid-flow mediated phenomenon for the development of new drugs and therapeutic strategies in these diseases.

Much of the current work is undertaken with support from the _____. The overall objective of the funded _____ project is to advance the current understanding of selectin and integrin biology by performing controlled experiments under shear and by developing rigorous bioengineering analysis techniques. These experiments are designed to provide the rationale for the development of selectin-ligand analogs that can be used for anti-inflammatory therapy, and to develop a fundamental understanding of factors regulating the time and shear dependent changes in selectin and β_2-integrin binding function. **The specific aims of the funded project are:**

Aim 1: To determine the critical biophysical and chemical parameters that control L-, E- and P-selectin mediated adhesion to their natural ligands and to synthetic carbohydrate ligand analogues.

Hypothesis 1: We hypothesize that effective neutrophil adhesion blockers directed against selectins, like many natural selectin-ligands, must also consist of an inner core-2 structure possibly with α1->3 fucose and α 2->3sialic acid linkages.

Hypothesis 2: We hypothesize that the amino acid residues Lys111, Arg97 and Arg46 in the lectin domain of L-selectin are critical for ligand binding under shear conditions.

Aim 2: To determine the factors that contribute to the differential function of β_2-integrin subunits (LFA-1 & Mac-1) during neutrophil adhesion, with time following stimulation.

Hypothesis 3: We hypothesize that the differential roles of LFA-1 and Mac-1 following stimulation play a critical role in controlling neutrophil adhesion rates.

We believe that we are making good progress with support from _____. Our group is establishing a niche in the combined application of computational/bioengineering modeling and experimentation with emphasis on vascular diseases. **While studies funded by _____ were underway, we observed that, fluid flow, besides affecting cell function as in the case of shear-induced platelet aggregation during thrombosis and mechanotransduction of endothelial cells, may also be regulating the function of soluble proteins in blood. We believe that there is little information on these aspects in current literature. *The reason for this lack of sufficient information may be attributed to the lack of tools to study such features in biomedical literature.* The current proposal addresses this gap in knowledge.**

From a career development perspective, while the emphasis of much of the previous work (_____) was on inflammation biology, our current focus is broader and it examines problems related to thrombosis and receptor biophysics also. These are both related issues since they are both influenced by fluid shear forces and the adhesion molecules participating are also often common. Further, while the previous work was largely focused on cell adhesion studies, the techniques used in our laboratory now are much broader and they include light and scattering, development of high-throughput assays in glycobiology, molecular biology and neutron scattering. The computational models we have recently developed are also significantly more sophisticated and novel compared to previous work carried out as part of the PI's Ph.D. and post-doctoral work.

The PI's appointment in an engineering school allows _____ to interact closely with colleagues in the area of material sciences and fluid mechanics. This has allowed us to apply non-traditional methodologies in the area of vascular biology. For example, SANS is typically used to study polymer properties and rheology. However, we demonstrate here that it is possible to use this technology to study biomolecules in solution and perhaps even the effects of fluid flow on these molecules.

We believe that the measurements we are starting to make in our laboratory are novel, and important in the disease/therapy context. **Further, for continued progress the PI needs to spend more time developing his creative ideas and pursuing careful experimentation in the laboratory. Further, he needs to learn and adapt experimental techniques applied in other research areas to address his interests in thrombosis. Such work will not be possible without funding support from a career development grant such as this**. **The prestigious nature of this award also motivates the PI to seek funding via this mechanism**.

If successful in the next five years, we will:

- Provide definitive proof that fluid shear-altered modulation of protein function plays an important role in controlling normal and pathological conditions in human health. While the focus of the current project is on vWF, we anticipate that pursuing these studies over the next five years may also lead to the discovery of other analogous processes in nature.
- Provide the community with quantitative experimental tools, mathematical models and new approaches to study hydrodynamic features regulating soluble proteins, cell-surface receptors and inter-cellular adhesion molecules.

Achievement of these objectives, in the long-term, will fully-establish the investigator as a leader in the study of the effects of fluid forces on vascular pathologies. Identification of unique motifs in proteins, that are shear-sensitive, may also lead to the design of drugs that function in a shear-dependent fashion. This has obvious implications to the treatment of vascular disorders associated with stenosis-like or other abnormal flow conditions. Finally, this project is also expected to boost biomedical research at _____ and help the investigator as he goes for tenure and beyond within the School.

C. Career Development Activities during Award Period

1) The Need for Protected Time*, PI's current responsibilities: teaching, administration and research*

At _____, the PI's job responsibilities include teaching, research and administration. _____. The following is a breakdown of the activities of the investigator:

Teaching: For a research scientist, teaching is no doubt a valuable asset since there is no better way to learn a research area than to teach someone else the same. Further, it allows important access to motivated graduate and undergraduate students who may be interested in research. On the other hand, teaching can be detrimental to the scientist if the teaching load severely limits the research effort or if the course material does not relate to research.

It is a requirement of the _____ (_____) at _____ that each faculty teach three-full courses each year. In this system, courses are NOT taught by a group of investigators, i.e. ALL lectures in a course are offered by a single faculty. Thus, over the last five years the PI has taught an array of courses:

- Cell and Molecular Bioengineering (_____, Undergraduate and Graduate level elective): 3 credits
- Biochemical Engineering (_____, Undergraduate and Graduate level elective): 3 credits
- Transport Phenomenon: Heat and Mass transfer (_____, Undergraduate Junior level course): 4 credits
- Unit Operations Laboratory 3 (_____, Undergraduate Senior level course): 2 credits
- Unit Operations Laboratory 4 (_____, Undergraduate Senior level course): 2 credits
- In addition, the PI presents lectures on leukocyte-endothelial interaction in the Advanced Cell Biology course (_____) at the _____.

On average, faculty are expected to teach 4.5 credits worth of courses each semester. Each credit equals one hour of contact time (i.e. class-lecture time) each week during the semester. 4.5 credits thus implies that 4.5 hours of teaching each week or over 130 hours of lecture time each year. Preparation time for each lecture (even if the course has been taught previously) includes planning/updating of lecture notes and photocopying handouts. Conservatively, this takes ~2 hours for each lecture hour. In addition, time is required for design of homework problems for each course and office hours. Even if teaching assistant help is available for any given course, grading of all exams has to be done by the instructor. **Thus, every semester, the PI spends at least 20-25 hours per week on teaching activities**.

Administration: The investigator is the co-director of the Center for _____ at _____. Responsibilities associated with this position include mentoring students seeking information about biomedical engineering programs or supervising those who are specializing in special majors in this field. The center also serves as a liaison for companies seeking to hire engineers or become involved in collaborative projects, and for other outside agencies. The center sponsors a seminar series, and publishes brochures and reports on biomedical engineering.

Additional administrative work at the department includes screening applications of candidates applying to join the department's Ph.D. program. The PI also sometimes participates in faculty search committees and administers some parts of the Ph.D. qualifying exams. **Participation in these committees and other administrative activities takes 5-10 hours each week**.

Research and Graduate student advisement*:* Research and grant writing is done in remaining time that is not taken up by teaching and administration. **In a 70-hour work week during the semester, thus, less than half the PI's time is allocated to research.**

2) **How will this proposal contribute to research career development ?**

The reason for the timing of this grant in the _____ is because, we believe that, we are in a position to make major scientific advancement and protected time is required to achieve this. The major objective of writing this grant is to dramatically improve the time available for research as detailed below. The time available will also provide a unique opportunity for the PI to update his knowledge in new methodologies in a structured fashion.

In planning these activities, the following constraints are kept in mind: i) It would be prudent for the PI to teach at least half- to one- course each year, since this will allow the PI to update teaching material in his area of research, i.e. Cell and Molecular Bioengineering. Teaching this course will also allow the PI to attract good graduate students into his group and not isolate him from the activities of other faculty in the department. ii) It would be good to continue as the co-director of the Center for _____ since this will allow the PI to shape the research environment at the school and this will directly benefit his research.

Based on the above rationale, funding of this grant will provide:

i. Complete relief from teaching for the investigator during the Spring semester each year. Reduction in teaching load during the fall semester, such that the overall number of courses taught by the PI is ½-1 course each year. This course will be in the area of specialization of the investigator.

ii. Moderate reduction in the administrative duties. While administrative responsibilities tend to increase in our department after tenure, the chair has committed to not place additional burden on the PI during the course of this grant.

3) Career Development/Training Activities During Award Period:

The training activities in this grant proposal are both structured and un-structured.

a) **Structured aspects of training: Formal Courses**

As part of the structured aspects of this training program, the investigator will undertake ~4 graduate level courses at the university and he will attend additional workshops. The selected courses are relate to the training/research goals of this proposal. These courses includes:

_____ **Topics in macromolecular structure** This is a three-credit course offered in the _____ department in the Spring semester each year. It exposes students to molecular simulation methods and advanced visualization techniques. While the PI is familiar with many of these methods on an *ad hoc* basis, the course will introduce this material in a structured manner.

_____ **Macromolecule Crystallography** This course is offered by the _____ department at _____ Institute (_____)[3] and _____ Department at _____ every Fall semester and it deals with crystallography methods. This is of interest to the PI since close to ~80% of all high-resolution structural information available today is from X-ray data and an understanding of the nuances involved in such measurements will be beneficial. Modeling strategies for X-ray data may also be translated to studies of neutron and X-ray scattering which are planned in Research Methods.

_____ **Magnetic Resonance** This course on NMR methods is offered by the _____ department at _____ every Spring and Fall. It is felt that this may be important since NMR can be used to study protein structure in solution at higher resolution than is currently possible using small-angle neutron and X-ray scattering.

Summer School on Methods and Applications of Neutron Spectroscopy. In addition to the above university courses, it is anticipated that the PI will attend Summer School on Methods and Applications of Neutron Spectroscopy offered each year at the _____. Each year, the course provides a general introduction to light and neutron scattering along with an extensive discussion of one-particular instrument. It is anticipated that participation in these courses will expose the investigator to new ways on thinking that are common to physicists, but not typical for researchers in biomedicine.

Surface Plasmon Resonance (SPR) training The SPR experiments conducted in this project will be performed on a Biacore-X surface plasmon resonance apparatus at the university. The PI will travel to the Biacore headquarters (Piscataway, NJ) during the first year of this grant to gain training on the use of this apparatus. It is thought that this 1-week training exercise is important since SPR is a major tool used in Aim 2 of this proposal.

The PI will also participate in instruction in the responsible conduct of research. A formal university wide course is offered in the spring semester of each year. This course will be undertaken in the initial year of this award. New information gained from this course will be disseminated to the graduate students in the PI's group and incorporated into the research program.

[3] _____ is located in downtown _____ and is ~10 miles from the PI's _____ location. Many faculty at _____ are affiliated with _____ and they offer joint courses at the university.

Graduate Research Ethics (_____) This course in responsible conduct of research is offered by the _____. It is taught jointly by the _____ School and the Department of _____ and is titled Graduate Research Ethics (_____). It meets once a week for a two hour session for one semester. This course addresses ethical issues in scientific research using lecture, video and forum discussion formats. The topics included are: the moral imperatives for biomedical research, understanding animal rights advocates' objections to the use of animals in research, ethics of transgenic animals, the necessity of animal research to human welfare, ethical principles for the use of human subjects in research, ethics of pre-andomization in clinical studies, ethical issues in experimental design and analysis, policies and practices for data ownership and retention in industry and university, intellectual property and conflict of interest, scientific misconduct-fraud and plagiarism, issues of genetic diseases, cloning and information, and the ethics of setting research goals.

b) Unstructured aspects of training: Self-study

As part of the unstructured aspects of this training program, the PI will be involved in self-study of material related to analysis of protein structure in solution. The study will address the following topics:

i. The PI will develop an understanding from first principal, of the fundamental basis of light, neutron and X-ray scattering methods. This is important to determine what sort of measuring tools would best suit our experimental needs.

ii. The PI will develop and adapt mathematical models that can be used to analyze experimental data on biomolecules structure in solution. Some examples of such data from our laboratory is presented in Preliminary Data

iii. The PI will determine to what degree statistical/computational theory can be developed to relate our experimental results on protein conformation under shear to primary, secondary, tertiary and/or quaternary protein sequence data available in the NCBI/pdb database. In the long run, we will thus use tools in the area of Bioinformatics to determine if any conserved structural motif exists in nature that allows proteins to act as force-transducers in response to non-physiological fluid shear.

The above unstructured training goals will be achieved thorough a combination of meetings with collaborators at _____ and elsewhere, reading and experimentation (See enclosed letters from collaborators).

In addition, the PI typically attends and presents results from his laboratory at 3-4 scientific meetings each year. These meetings are organized by the Federation of American Societies for Experimental Biology (FASEB), American Heart Association (AHA), Vascular Biology (NAVBO), The International Society on Thrombosis and Haemostasis (ISTH), Gordon/Keystone Conference, Biomedical Engineering Society (BMES) and American Institute of Chemical Engineers (AIChE). _____ also presents seminars on _____ work at other schools and attends grant-review panels. Finally, the PI also reviews journal articles for several journals and serves on the editorial board of _____. There is also an active journal club in the department on Biomedical Engineering and a departmental seminar series. These represent unstructured training opportunities that will enhance the learning and career development aspects of this proposal.

c) Mentorship and bi-annual review

As part of the training program, a mentorship program which will be setup where the PI will meet with the chair of the department to asses progress on this grant at six month intervals. This meeting will determine if any corrective actions need to be taken to remedy shortcomings and to improve the impact of this award.

5. Environmental and Institutional Commitment to Candidate:

Please see the following letter from the Department Chair highlighting the resources available in the PI's laboratory and collaborators at the University at _____. Details of specific equipment available for this project are listed in the Resources page. This letter also contains details on the nature of reduced teaching and administrative loads for the PI.

6. Research Plan

A. Statement of Hypothesis and Specific Aims

In recent experiments, we applied our quantitative experimental strategies to study shear-induced platelet activation. Here, we observed using static and dynamic light scattering, that fluid shear in addition to regulating cell function, may also regulate bio-molecular structure and function. Application of shear forces in these studies lead to the self-association or aggregation of blood plasma protein vWF. ***The overall goal of this project is to determine if plasma vWF responds to applied fluid forces by altering its molecular structure, and to gain new mechanistic insight into the nature of vWF self-association***. The reason for choosing the K02-grant format for funding is because the PI needs to develop new tools and understanding to pursue the above goal. _____ also wishes to formalize training in some non-traditional methods, which he currently uses on an *ad hoc* basis. These methods include light, X-ray and neutron scattering, surface plasmon resonance and molecular simulation of biomolecules. Some of these tools are typically used in the areas of material science to study polymeric and liquid crystal structure under fluid shear. We propose here that such methods may also find important applications in studies of vascular biology. The specific aims of the project are:

Aim 1: to determine the shear conditions under which vWF may self-associate and undergo conformational changes.

We test the hypothesis that soluble proteins and biomolecules exposed to blood circulation experience hydrodynamic forces that can affect their structure and function. These studies examine the response of proteins to fluid forces in real-time using light, neutron and X-ray scattering. Specifically, in some experiments we use light scattering to distinguish between the contributions of shear rate and shear stress in inducing vWF self-association under physiologically relevant conditions. In other runs, we apply Small-angle neutron scattering (SANS) to study the structural changes of vWF that is dissolved in a deuterated buffer under shear in a Couette viscometer. Taking advantage of the property of X-rays to distinguish between proteins and aqueous media, which have distinctly different electron densities, we also use Small-angle X-ray scattering (SAXS) to confirm/enhance the findings of the SANS experiments. Finally, with the objective of establishing custom instrumentation in our laboratory to study the response of proteins to shear flow, we propose to build Rheo-optics capability at _____ that will allow us to perform real-time light scattering measurements of biomolecules under shear.

Aim 2: to quantify the kinetics and shear-dependence of plasma vWF self-association in a physiologically relevant milieu using surface plasmon resonance (SPR).

We test the hypothesis that vWF may self-associate upon application of shear, in mixed systems with other human proteins and under experimental conditions that mimic the physiological milieu. In one set of experiments performed with purified human vWF, we apply surface plasmon resonance to quantify the forward and reverse rates of vWF-self association over a range of shear rates, under conditions where the substrate bears immobilized collagen. Here, we also determine the pH and cation-requirements for such self-association. In other runs that aim to more closely mimic the physiological milieu, we perform experiments in mixed systems with other proteins found in blood plasma, to determine their role in either augmenting or decreasing the amount of vWF self-association. Mass spectroscopy and/or HPLC will be applied, in selected cases, to determine if other proteins are bound to the self-associated vWF in the SPR runs.

Aim 3: to determine unique structural features in biomolecules that make them act as “force-transducers”.

In this aim, we begin to establish computation methods using molecular simulations and other techniques to study the response of proteins, specifically the A1 domain of vWF, to shear flow. Upon comparison with results of mutant vWF simulations and data from Aim 1 and 2, we will *examine if the structural changes due to gain-of-function mutations are similar to changes occurring under shear*.

B. Background, Significance, and Rationale

Fluid flow regulates vascular function

Flowing blood plays an important role in both initiating and regulating biological processes in circulation. For example, high shear stresses have been shown to contribute to platelet activation, and subsequent aggregation and secretion (16, 21). Gene expression and protein synthesis in endothelial cells is also altered following mechanotransduction (22). In addition to controlling cellular activation rates in the above cases, hydrodynamic shear also controls the rates of cell-cell collision, deformation, receptor-ligand bond formation and adhesion. In a prominent example, fluid shear has been shown to allow optimal L-selectin mediated leukocyte rolling only above a minimum ‘threshold’ shear rate (9, 23). Finally, there is growing evidence to indicate that flowing blood alters conformation of plasma protein von Willebrand Factor (vWF). In one example, a metalloprotease ADAMTS13, the deficiency of which causes thrombotic thrombocytopenic purpura (TTP) (24), has been shown to cleave vWF under shear but not static conditions (25, 26). We have also shown that fluid forces can induce the self-association of plasma vWF (16).

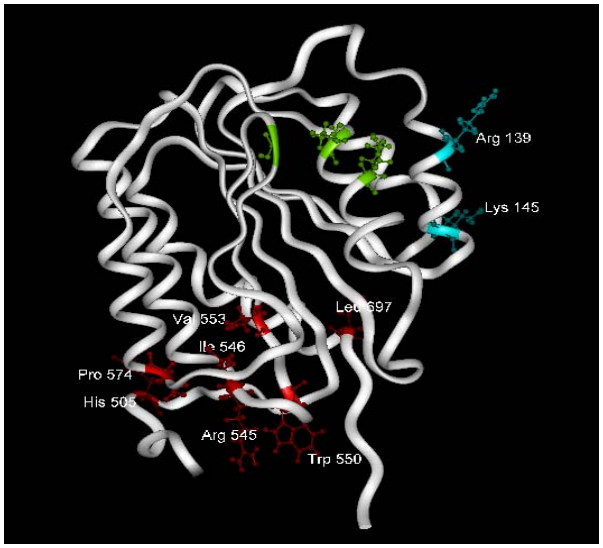

FIG. 1: Figure depicts the A1 domain of vWF. Spatial position of vWD Type IIb mutants in the A1 domain are indicated in red.

Our interest in von Willebrand Factor (vWF)

Much of this proposal deals with a large plasma protein called Von Willebrand factor (vWF) (21, 27-29). This molecule occurs both as a monomeric unit and in multimers with molecular weight in the range from ~500-10,000kDa (see western blot in Prelim Data). Under normal conditions, vWF does not bind blood platelets spontaneously. However, under vascular injury situations, when vWF adheres to extra-cellular matrix proteins exposed on the denuded vascular endothelium, the affinity of the immobilized vWF for platelet surface-receptor GpIb is enhanced, and this allows platelet recruitment and subsequent aggregation at sites of injury (30). The domain of vWF that binds GpIb is called the A1 domain (Fig 1) (31). The size of

platelets, and the strength and kinetics of GpIb-vWF interactions under shear are such that even under the high fluid velocities of the arterial circulation, platelet adhesion occurs.

The observation that application of high fluid shear forces alone on platelets in suspension in cone-plate viscometers causes activation of the platelets and subsequent aggregation, following shear-induced binding of plasma vWF to platelet GpIb/IX/V (32, 33) suggests that vWF-GpIb binding may be facilitated by fluid shear (17) and this binding may trigger cell activation and subsequent aggregation (34-36). In this regard, we have shown that the binding of vWF to GpIb itself is not sufficient for cell activation; fluid forces must be applied on the GpIb-vWF bond to trigger this signaling process (16). It is suggested that such shear-induced platelet aggregation (SIPA) processes may play a role in the pathogenesis of vascular diseases since platelets from patients with acute mycocardial infarction (37, 38) and cerebral ischemia (39, 40) display enhanced SIPA *in vitro*. **Studies of the interplay between platelet GpIb, plasma vWF and shear in regulating cell function are thus important**.

Besides fluid forces and vascular injury discussed above, a family of point mutations in vWF called vWD Type IIb mutations also increase the affinity of vWF for GpIb. The structural changes induced by these *gain-of-function* mutations are discussed at length in Preliminary Data. The *hypothesis that structural changes due to gain-of-function mutations are similar to changes occurring under shear is intriguing*, and we will address this during the course of this project.

A recent report has suggested that besides binding to blood platelets, vWF molecules also bind surface-immobilized vWF, and that this “self-association” may contribute to vascular injury (41). We have also shown using static and dynamic light scattering that vWF aggregation (or self-association) is mediated by hydrodynamic shear forces (16). The potential role of vWF self-association in thrombosis is not clear and we address this in some detail during this proposal.

The **specific questions we ask are**:

i) How does fluid shear alter the solution conformation of vWF? Does vWF align itself in the direction of flow as do certain polymers (42)? These questions will be examined in **Aim 1** using SANS, SAXS and light scattering.

ii) At what rate does vWF self-associate in a physiological milieu where other blood proteins are present? Does the presence of other blood proteins augment or decrease this self-association rate? How does the binding of vWF to a substrate depend on applied shear? These issues will be addressed using surface plasmon resonance in **Aim 2**.

iii) Are hydrodynamic forces applied on blood proteins under physiological and pathological conditions sufficient to induce protein conformation changes? If so, is there a unique structural motif that allows these proteins to act as ‘force tranducers’? Novel computational approaches are taken to address these questions in **Aim 3**.

We believe that the above questions are significant from the perspective of biology, medicine and biophysics, and hence we seek support from the NIH for this project.

C. Preliminary Studies and Any Results

1) Nature of hydrodynamic forces applied on inter-cellular bonds, soluble molecules and cell-surface proteins (in review)

Currently, well-defined solutions exist for the estimation of forces applied on cells tethered onto substrates (43). Such models have aided estimation of the kinetics (largely off-rates) of receptor-ligand bonds in the context of selectins and integrins (44-46). Well-defined theories also exist for the estimation of forces between two equal size particles e.g. homotypic aggregates of either two neutrophils, two platelets or two red blood cells (14, 47-49). We have recently extended this methodology for calculation of forces between heterotypic aggregates in suspension ((19), see Appendix).

This is an important development since, heterotypic aggregates e.g. neutrophil-platelet and platelet-tumor aggregates play an important role in the progress of cardiovascular diseases and cancer metastasis, yet estimates of the magnitudes of forces applied on bonds between such aggregates are currently unavailable. In this manuscript, we show that while this mathematical approach can be used to estimate the hydrodynamic forces applied on intercellular bonds between neutrophil-platelet, neutrophil-neutrophil, neutrophil-transfectant, platelet-platelet and platelet-tumor aggregates, **it can also be applied to estimate the magnitude of forces on micro-domains of cell-surface receptors (like L-selectin on neutrophils or GpIb on platelets) and soluble molecules (like vWF)**.

In the long run, we anticipate that such analysis will allow us to: i) translate data from single molecule studies (e.g. atomic-force microscopy measurements) to predict ensemble behavior in suspension, ii) design appropriate *in vitro* experimental systems to apply the range of hydrodynamic forces that are relevant *in vivo*, and iii) understand the fundamental mechanisms of fluid flow initiated biological phenomena.

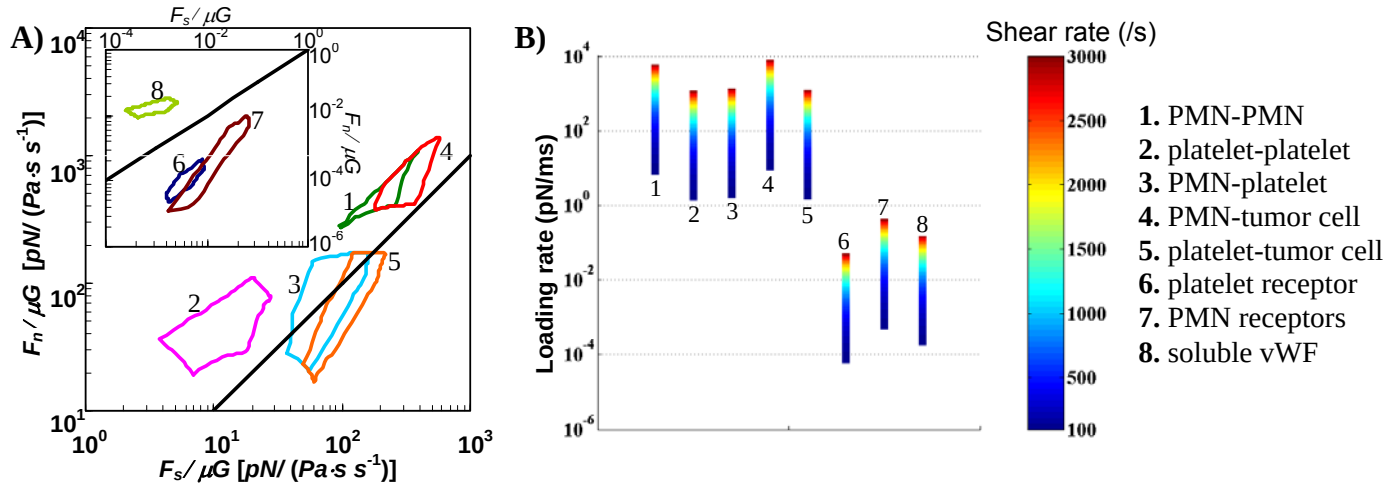

FIGURE 2. A) Normal and shear forces. The regions indicate the outer bounds of the hydrodynamic forces for the following cases: 1. PMN-PMN (dark green), 2. platelet-platelet (pink), 3. PMN-platelet (light blue), 4. PMN-tumor cell (red), 5. platelet-tumor cell (orange). Regions in the inset indicate ranges of smaller forces for: 6. platelet receptors (dark blue) 7. PMN receptors (brown) and 8. soluble vWF molecule (lime). The limits on the forces were obtained by using the geometric features of the cell/molecules from published data (see full-paper for details). All force data is shown normalized by μG. Multiplying the values in the chart with the viscosity (in Pa·s) and the shear rate (in s^{-1}) yields the applied force in pN for each case. Bold black line corresponds to $F_n/\mu G = F_s/\mu G$. If the force for a given object lies above this line, the normal force dominates. Otherwise, the shear force is dominant. Normal forces are high for 1-4, 8, and shear forces dominate for 5-7. **B) Force loading rates.** Time periods of rotation were computed for the above cases (1 to 8 above). These calculations were used with the force estimates (panel A) to obtain the maximum force-loading rates (panel B) over a range of shear rates. Media viscosity is set to 1cP and the particles are subjected to a linear shear of magnitude G.

In Figure 2, we present the range (or limits) of forces and force loading rates applied in the above cases under physiologically relevant conditions observed *in vivo* and in *in vitro* experiments. For these calculations, cells and aggregates are subjected to a linear shear field of magnitude G in fluid with viscosity μ.When such a flow is imposed on cells/aggregates, the particles tumble or rotate in the flow. Depending on their orientation with the flow field, the inter-cellular bonds and cell-surface receptors may feel either a normal (either tensile or compressive) or tangential (i.e. shear) force. Depending on the size of the participating cells and adhesion molecules, the magnitude of force applied and the force loading rates varies (Fig. 2): **i**) The maximum force applied on neutrophil homotypic aggregates (case 1) is greater than

that on neutrophil-platelet aggregates (case 3) by ~7 fold (Fig. 2A). Thus, altering cell size may dramatically alter the requirement of adhesion molecules required for cell binding. **ii**) Surprisingly, the peak forces (~0.1pN) and loading rates (~100-1000pN/s) on GpIb and vWF-like molecules are of comparable magnitude. The forces and loading rates on these cell-surface and soluble proteins are orders of magnitude lower than that applied on inter-cellular bonds (Fig. 2B). **iii)** While normal forces are ~3 times larger than shear forces in the case of neutrophil doublets, shear forces may be the dominant force in other geometries.

Throughout this project, we propose to utilize the above calculation scheme to estimate the magnitude of force applied on vWF in our experiments under shear. This calculation method will also be used to compare the magnitude and nature of forces applied on vWF with other cases of physiological interest where receptor-ligand bonds are stressed or when cell-surface receptors undergo functional changes upon application of shear. NHLBI grant RO1 HL63014 is allowing us to examine a number of such cellular systems, especially those involving the selectins, and thus the studies on vWF proposed here complement other studies in our laboratory.

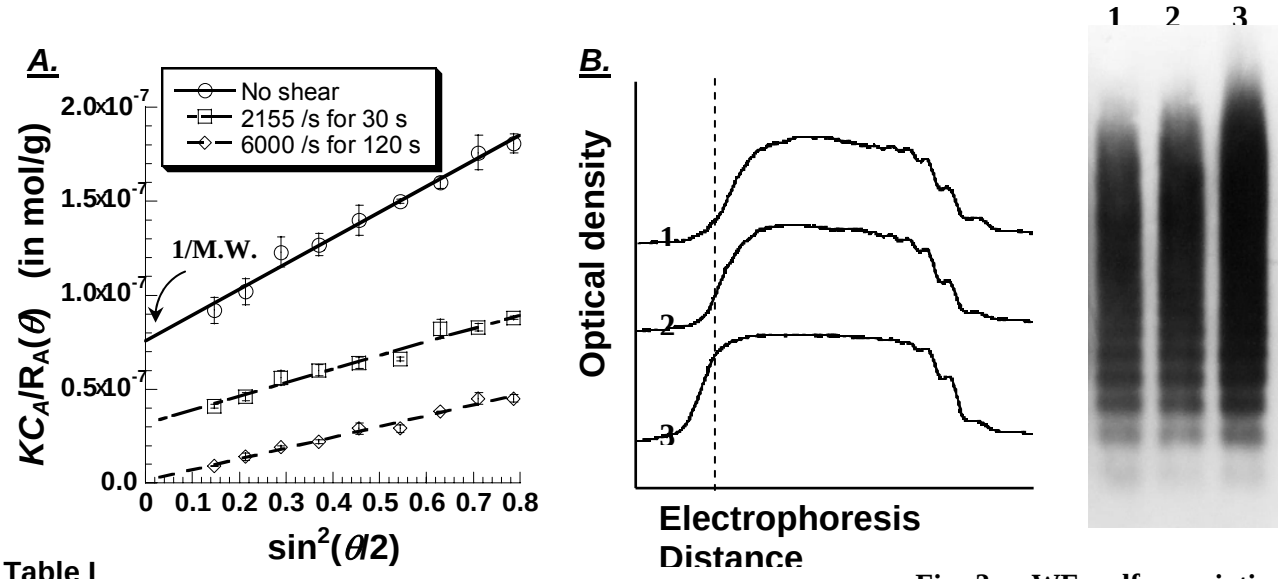

Table I

Shear applied	Molecular weight, *M* (Da)[#]	Radius of gyration, R_g (nm)[#]	Hydrodynamic Radius, R_h (nm)[§]
No shear	9.6 x 10^6 (± 3.7x 10^6)	97.1(±29.6)	68.2(±19.2)
2155/s for 30s	25.1 x 10^6 (± 6.9x 10^6)	113.8(±27.0)	91.3(±22.9)
6000/s for 120s	695 x 10^6 (± 152x 10^6) *	394.8(±117.7)	466.4(±95.6) *

Fig 3. vWF self-association under shear. **A.** Static light scattering data showing increase in vWF molecular weight (M.W.) upon shear application. Y-intercept in panel **A** equals 1/M.W. Data is representative of two runs with same vWF preparation/isolation. **B.** Western blot and densitometry analysis show that isolated vWF exists both as unimers and as multimers with upto 20-25 units (trace **1**). Application of shear at either 2155 for 30s (**trace 2**) or 6000/s for 120s (**trace 3**) results in the formation of multimers of size greater than 20mers. These larger molecular weight events lie to the left of the dashed line in panel **B. Table I**. Summary of results from light scattering experiments show that hydrodynamic shear causes a statistically significant increase in vWF size. Data are Mean (± SEM) for 3 vWF isolations. [#] data from static light scattering analysis. [§] data from dynamic light scattering analysis. [*] Significantly different from the unsheared sample.

2) *Purified vWF self-associates upon application of fluid shear (_____)*

We recently reported on the self-association of plasma vWF upon application of hydrodynamic shear (16) (Fig. 3). For these studies, human vWF was isolated from blood cryoprecipitate obtained from the _____. Both unsheared samples and samples subjected to shear in a cone-plate viscometer were examined using light scattering. Static light scattering analysis (Fig. 3A) shows an increase in vWF

molecular weight upon shear application. The unsheared purified vWF in this panel has a molecular weight of 13.3×10^6Da and a radius of gyration of 71.7nm. Subjecting this vWF to shear at 2155/s for 30s resulted in an increase in MW to 32.0×10^6Da and R_g to 82.0nm. Shearing samples at 6000/s for 2mins, further increased MW to 847.5×10^6Da and R_g to 341.3nm. The data is reconfirmed using Western blot analysis, where large vWF aggregates are observed in the sheared samples (Fig. 3B). It is noted here that addition of 0.1% SDS to vWF aggregates within seconds causes partial, albeit incomplete, dissociation of the vWF aggregates suggesting that these aggregates are a result of non-covalent interactions (see paper in Appendix for details). Higher molecular weight fractions in western blot are those vWF aggregates that are resistant to SDS treatment. The data in Table I for 3 different vWF isolations/preparations demonstrates a statistically significant increase in vWF size upon application of shear. The physiological significance of this observation will be studied in more detail during this project.

3) Small-angle neutron scattering of vWF
(_____)

SANS is used to study the structure of molecules in solution based on the contrast between the protein and the deuterated solvent. Thus, for our experiments performed at _____, we purified human vWF from blood cryoprecipitate of different donors and dialyzed the samples extensively against deuterated buffer. These samples were placed in the path of a cold-neutron beam and the scatter data was collected.

Preliminary studies (Fig. 4) demonstrate that SANS experiments performed using the NG3 instrument at _____ (_____) can allow us to study the structural features of vWF. The figure compares our findings from SANS with light scattering and Western blot analysis for vWF from two different donors. As seen in the western blot, vWF in solution exists as multimers comprising of 1-50 500KDa protomer units (Fig 4A). Western blot analysis reveals that the vWF sample purified from the B^+ donor (B^+ vWF) has a larger proportion of very high MW species compared to the O^+ vWF sample. Light scattering (Fig. 4B) confirms these findings and places the weight-averaged molecular weights of the B^+ and O^+ samples at 63,000KDa and 30,000KDa respectively. Modeling of the absolute SANS intensities at low-scattering vector (Q) agree with western blot and light scattering analysis that the molecular weight of B^+ vWF is greater than that of O^+ vWF. Further, analysis was performed with an ellipsoid form factor incorporating results from vWF concentration and molecular weight measurements. The analysis reveals, for the first time, that in solution vWF remains as an extended molecule with a rod-like shape with a minor axis radius of ~15nm and a major axis radius of ~100nm (Fig. 4C). At high-Q, we also observed 5nm globular domains. The studies revealing the shape of vWF are important since it is still controversial if vWF exists as a rod shaped molecule, or if application of hydrodynamic forces cause it to exhibit an elongated shape. Our studies above suggest that the molecule is predominantly in an elongated form in solution. Further analysis will be performed during the course of this project to model the data over all Q-ranges by representing vWF as a string of beads, and also account for the polydispersity in the system. The model will provide novel information on the specific organization of the globular domains that form a full-length vWF multimer.

We wished to determine if SANS could be applied to study structural changes in vWF. Thus, we prepared deuterated Guanidine-DCl according to published protocol (50) and characterized it using Fourier Transform Infrared spectroscopy (FTIR) and NMR. This salt which partially denatures proteins, and which allows the action of the ADAMTS13 metalloprotease on vWF was added to the vWF (Fig. 4D). As seen, Guanidine-DCl causes structural changes in vWF that could be detected using SANS. These changes caused the aspect ratio and radius of gyration of vWF to increase.

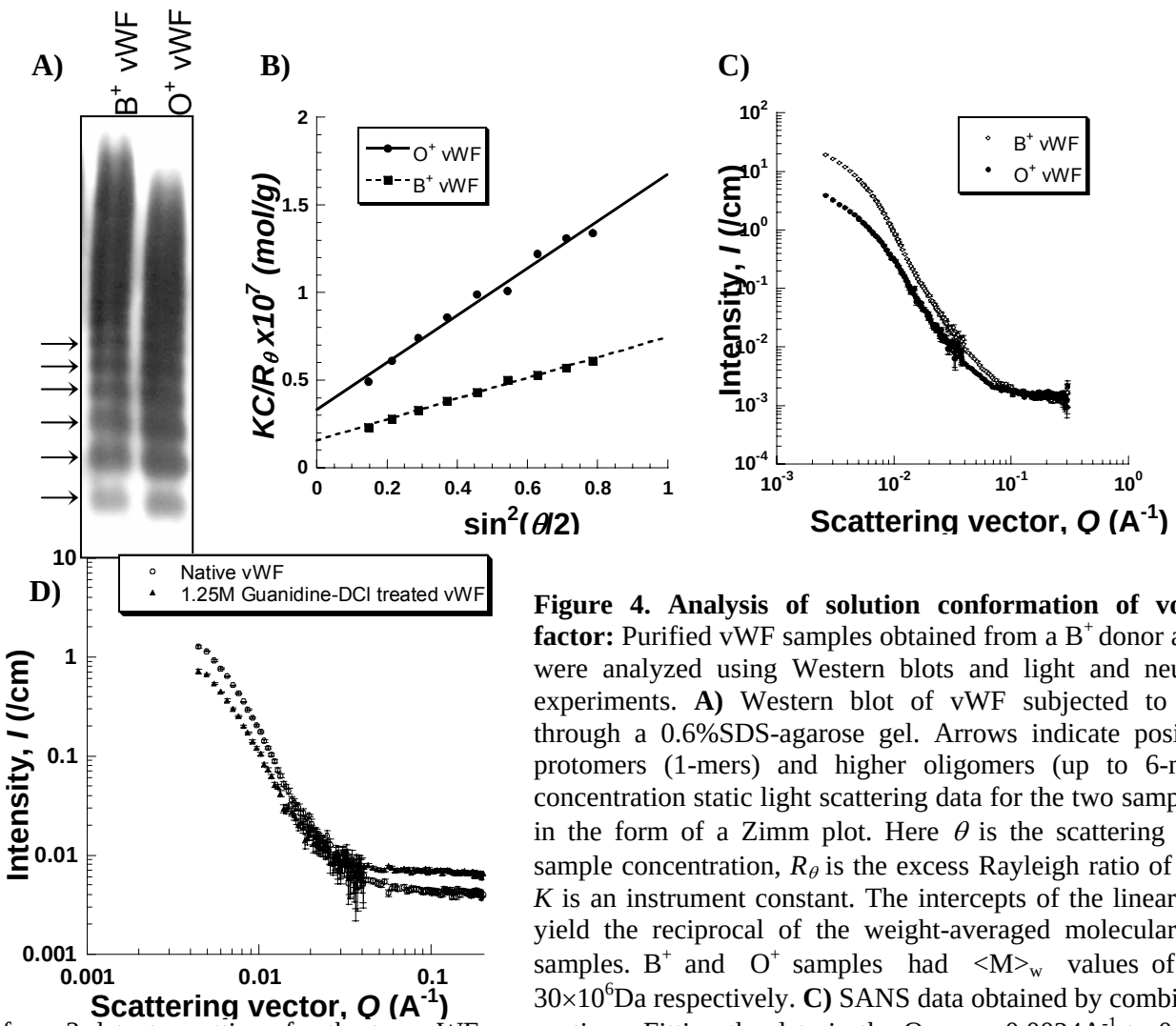

Figure 4. Analysis of solution conformation of von Willebrand factor: Purified vWF samples obtained from a B^+ donor and an O^+ donor were analyzed using Western blots and light and neutron scattering experiments. **A)** Western blot of vWF subjected to electrophoresis through a 0.6%SDS-agarose gel. Arrows indicate positions of single protomers (1-mers) and higher oligomers (up to 6-mers). **B)** Low concentration static light scattering data for the two samples is presented in the form of a Zimm plot. Here θ is the scattering angle, C is the sample concentration, R_θ is the excess Rayleigh ratio of the sample and K is an instrument constant. The intercepts of the linear fits to the data yield the reciprocal of the weight-averaged molecular weight of the samples. B^+ and O^+ samples had $<M>_w$ values of 63×10^6Da and 30×10^6Da respectively. **C)** SANS data obtained by combining results from 3 detector settings for the two vWF preparations. Fitting the data in the Q-range $0.0024A^{-1}$ to $0.01A^{-1}$ with an ellipsoid form factor reveals that vWF is a prolate (rod-like) ellipsoid. **D)** Addition of deuterated Guanidine-DCl revealed changes in conformation of vWF at both the low and high Q-ranges.

During the course of this grant we will extend the above studies by placing a Couette shear-cell in the path of the neutron beam in order to determine, in real-time, the response of this molecule to fluid forces.

4) Molecular simulations of Type IIb vWD (von Willebrand disease) reveal changes in the binding pocket of GpIb

(unpublished data)

While X-ray data is now starting to provide limited 'snap-shots' of the vWF structure, the detailed dynamic mechanism for the conformational change is lacking. In this regard, molecular simulations provide a complementary tool to obtain information that is not yet possible to access using experimental methods. With this in mind, we have initiated a collaboration with Dr. _____ in the _____ Dept. at the university to develop computational-biology based models of proteins under shear. As a starting point we are examining vWF using CHARMm (Chemistry at HARvard Macromolecular Mechanics). The availability of a super-computing facility on campus is a major advantage for pursuit of this line of work.

The A1 domain of vWF is the motif that binds platelet GpIb. The first step towards efficiently generating stable three-dimensional structures for mutant proteins using molecular dynamics is the computation of an equilibrated wild-type structure that accurately reflects the behavior of the protein in solution (**Fig. 5**). To obtain this, we employed the crystal structure of the human von Willebrand factor (vWF) A1 domain from the Protein Data Bank (31). We then obtained an equilibrated wild-type structure using molecular dynamics (MD). Briefly, the crystal structure was soaked in 3 layers of explicit water molecules. During the equilibration simulation, the protein was initially constrained in order to prevent unfolding that could result from the sudden introduction of kinetic energy into the system. The constraints were gradually removed and the simulations were continued until the structure had stabilized. This involved 1.6 ns of MD. *The fluctuations of the individual residues in the equilibrated structure compare well with the values calculated from X-ray diffraction experiments* (Fig 5)*. Thus, we have obtained an equilibrated structure that serves as an accurate starting point for the proposed studies.*

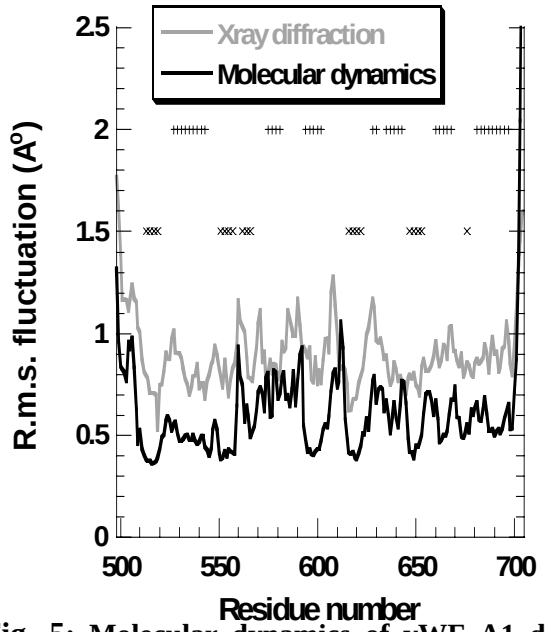

Fig. 5: Molecular dynamics of vWF A1 domain. Molecular dynamics was performed to obtain the wild-type vWF A1 domain. Comparison of root mean square fluctuation data obtained from molecular dynamics with that calculated from X-ray diffraction data demonstrate that we have a good starting point for our mutation/shear simulations. Positions of helices and sheets are indicated using '+' and 'x' symbols respectively.

In limited mutation studies, we have also recently employed molecular dynamics to obtain the structure for the vWD type IIb mutant Ile 546 -> Val (abbreviated I546V) (red residue in Fig 1, data not shown). This mutation in patients results in an enhanced binding of vWF for GpIb, and this results in thrombocytopenia and prolonged bleeding times *in vivo*. In these studies, we compared the mutant and wild-type structure with regard to: i) the dihedral angles of the peptide backbone and ii) root mean square (r.m.s.) displacement of residues with respect to the wild-type structure. Our results demonstrate that single mutations at the bottom of the vWF structure cause some re-orientations in the dihedral angles of the bonds. These re-orientations propagate over a 27 Å distance from residue 546 to the GpIb binding pocket located near residue Gly 561 (31, 51, 52). Our simulations reveal that the mutation I546V induces the peptide plane Gly561-Ser562 to re-orient itself on average by nearly 30° (data not shown). Interestingly, in one of the structures published for

vWD Type IIb mutation (also I546V) (53), researchers have observed a 107° dihedral angle change in the Asp 560-Gly 561 plane. Although, our result does not quantitatively match this value, it seems to demonstrate functionally significant changes starting to manifest themselves in the structure of the protein. Longer timescale simulations will be performed during the course of this project to further confirm these findings. Interestingly, analysis of the dihedral angles also shows significant changes in residues 631, 633 and 634. These torsion angle changes lead to a significant displacement in the helix 4 (628-630). The possible significance of these changes to the function of the A1 domain will be examined during this project. Further, we will attempt to simulate the structural changes in the A1 domain upon application of shear to address the hypothesis that structural changes due to mutations are similar to changes occurring under shear.

D. Research Design and Methods

Although we have established that vWF self-associates under shear flow conditions, we are yet to carefully establish the physiological conditions under which this may occur. Also, it is not yet clear if hydrodynamic flow can cause changes in the conformation of vWF micro-domains under shear. This latter feature may be an important factor controlling vWF-GpIb binding and platelet activation rates. We address these issues in the following aims.

1) (Aim1): Studies using light, neutron and X-ray scattering: Can shear forces alter protein conformation in suspension?

i) Light scattering to distinguish between the contributions of shear rate and shear stress in inducing vWF self-association under physiologically relevant conditions

This aspect of the project will distinguish between the contributions of shear rate and shear stress in inducing vWF self-association using a cone-plate viscometer to apply defined shear conditions, and using light scattering for analysis of vWF-aggregation rates. For these runs, vWF will be isolated from human blood cryoprecipitate obtained from the _____ as we and others have described elsewhere (16, 54, 55). Prior to experimentation, the sample will subjected to centrifugation at 40,000g for ~1 hr. to remove any large aggregates that may be present. We have already carried out similar experiments with vWF (16) and we have experience on the preparation and handling of the samples. This purified molecule will be subjected to a range of shear rates (1000-9000/s) for 30s using a cone-plate viscometer. The aggregation of the protein will be measured using static and dynamic light scattering to analyze the vWF size-distribution after shear. Quantitative analysis of data based on i) a particle adhesion model (1, 56), and ii) force calculations ((19), Fig. 2) will allow us to clearly distinguish between the roles of protein-protein collision and shear forces in mediating the self-association.

The shear stress τ for Newtonian fluids is directly proportional to the applied shear rate according to $\tau = \mu G$, where μ is the media viscosity. In some runs, to distinguish between the contributions of shear rate and shear stress, media viscosity will be altered by the addition of dextran at a final concentration of 1.5% w/v. We have used similar protocols in other studies (9, 19). Here, we wish to establish the critical shear rate or shear stress condition required for vWF self-association. We are aware that this last set of experiments may be difficult since the presence of dextran may complicate our estimates of vWF molecular-size using light scattering. That being the case, western blot analysis may be used to obtain qualitative information on the role of shear stress and dextran in inducing vWF self-association.

In control experiments, we will shear purified human plasma fibrinogen and serum albumin to determine if similar shear-induced self-association behavior is observed with other blood proteins also. Frankly, we do not anticipate any such behavior with these proteins since to our knowledge, shear-induced changes have been reported for vWF only. Also, in preliminary SANS experiments carried out in a couette shear-cell, we did not detect fibrinogen self-association or conformation change when this protein was sheared upto a shear rate of 1000/s (data not shown).

ii) Small angle-neutron scattering (SANS) to determine protein conformation under shear

We have applied neutron scattering spectroscopy (SANS) to analyze the shape of vWF under static (but not shear) conditions (Fig. 4). These studies also reveal that SANS is capable of resolving conformation changes in vWF induced upon addition of Guanidine·HCl. In this aim, we will determine if we can detect similar conformational changes in vWF when this protein is subjected to defined hydrodynamic shear in a couette shear-cell.

The shear cell we will use is built at the _____ laboratories and is described in detail elsewhere (57). Briefly, the gap between the inner stator and outer rotor in this device is 1mm and shear rates upto 1500/s can be applied on the sample before the onset of turbulence. This device has been specifically constructed to interface with the SANS NG-3 instrument so that the neutron beam may pass through the device as it is being mixed. We have already performed control experiments using this device for fibrinogen to assess the feasibility of the experiment with biological proteins.

During the course of this project, we will extend the above measurements to study vWF conformation and aggregability upon shear. This approach allows us to study vWF structure in real-time under shear, without the introduction of fixation artifacts that are common to recent Atomic Force Microscopy studies (58, 59). The PI has well-established collaborators at _____ and _____ who will help with this aspect of the research (see support letters). Two types of runs will be performed: (i) time-resolved studies that will assess the vWF sample at varying times after the initiation of shear, and (ii) studies that assess vWF under static conditions at specific time intervals, after sample shearing. Thus, SANS will allow determination of the shape and dimensions of vWF during shear, and following cessation of shear. In these experiments, we expect to observe the alignment of vWF in the direction of flow following the initiation of shear. This alignment may be accompanied by an elongation of vWF, that is perhaps followed by unfolding and/or aggregation. The conformational changes at the molecule level will be reflected in shape transitions, e.g., transition from ellipsoid to coil, or to more elongated ellipsoid in the reciprocal space analysis, and in a change in distance distribution function obtained by the GIFT analysis (60).

In the SANS experiment, it is possible to distinguish between vWF self-association and biomolecular shape by performing scattering analysis as a function of the scattering vector (Q). Aggregation will result in an increase in scattering at low Q, while changes in the conformation of domain will be observed at high Q. Further, shape changes like the elongation of molecules will be reflected by changes in the form factor of scattering. Any ambiguity in the above data analysis can be resolved by addition of deuterated SDS during the above experiment, which prevents vWF self-association as we discussed in the Preliminary Data section.

In limited experiments aimed to aid the above modeling efforts, we may use the recombinant A1 domain of human vWF to study changes in the conformation of this domain following Guanidine HCl treatment. We are interested in this molecule since the vWF A1 domain is the functional domain that contains the binding pocket for platelet GpIb. Further, while the binding of vWF A1 domain to platelets can trigger cell activation, the self-association of vWF is not due to homotypic A1 domain binding interactions (41). During the course of this project, this recombinant molecule will be obtained from one of the PI's current collaborators. The X-ray crystal structure of the A1 domain is known and we will be interested in comparing the molecular shape from our study in solution with the crystal structure.

iii) Small angle X-ray scattering (SAXS)

We will conduct experiments using small-angle X-ray scattering (SAXS) and compare it with SANS studies to determine if this technology would be appropriate for studies of vWF. The SAXS studies will be performed at the _____ (_____) facility at the _____ (_____),_____ Laboratory. _____We are interested in SAXS for the following reasons:

i) The electron flux at APS is many orders of magnitude greater than the neutron flux rate at _____. Thus, we anticipate that the time taken to assay the structure of our protein samples will decrease from hours (as in the current case of SANS) to minutes.

ii) The SAXS beam is narrow compared to the SANS beam and thus, the amount of precious protein sample used for the SAXS methods will likely be smaller.

iii) The length scales that we can study using the BioCAT 18-ID beamline ranges from 0.5 to 1570 Armstrong (corresponding to a Q-range from 0.004 to 1.25Armstrong^{-1}). In addition to covering the length scales that are observed using SANS (where the Q-range in our experiments varies from 0.003-0.3 Armstrong^{-1}), this instrument also offers higher resolution at smaller length scales. Thus, we may be able to probe smaller features of the vWF.

iv) The analysis methods for SANS can be adapted to SAXS experiments also.

A potential drawback of the SAXS instrument is the radiation damage to the protein due to the beam which limits the post-experiment analysis that can be performed on the samples. This issue will be studied in detail during this project. Data from the SANS experiments which we have already collected may be used to standardize the SAXS runs. Shear experiments can also be performed both using SANS and SAXS.

iv) Rheo-optics for study of biomolecules under shear.

While SAXS/SANS allows us to study protein structure down to Q-values of 0.003 Armstrong^{-1}, light scattering can be used to study the structural feature at lower Q-values, which correspond to larger objects like self-associated vWF complexes. For this reason, we believe that it would be useful to setup rheo-optics methods in our laboratories, which will allow real-time light scattering analysis of our biomolecules under shear.

Development of such instrumentation in our laboratories will require the design and construction of a quartz couette shear-cell for either a VT550 viscometer (from Haake, Inc. which is available in the PI's laboratory) or a ARES-RFS (manufactured by Rheometrics, Inc. available in the laboratory of our collaborator, _____). The viscometer will be placed in the path of the 514nm laser beam that is part of the Brookhaven light scattering Goniometer in the _____ laboratory (BI-200SM Ver.2.0, Brookhaven Instruments, Holtsville, NY). We plan to set this instrument up for static light scattering, though it may be extended for dynamic light scattering in the future.

Development of such rheo-optics instrumentation has gained momentum recently, and it is finding application in studies of polymer conformation in purely extensional flow (42), in small-angle light scattering application of liquid crystal polymers (61-63), and in dynamic light scattering studies (64, 65). A textbook on rheo-optics has also been published (66) which gives us confidence that such methods can be implemented. In our view, setting up of such instruments will be challenging and will require additional grant support. It will also require a detailed understanding of light scattering methods from first principle as described in the training section of this proposal. Notwithstanding these difficulties, availability of such unique instrumentation in our laboratory will also help us to better design our SAXS and SANS runs prior to traveling to the experimental site for allocated beam-time. In the long run, it will also provide us with unique instrumentation and capabilities for future work.

2) (Aim2): Studies using surface plasmon resonance: Do vWF unimers self-associate upon application of shear, in mixed systems with other human proteins and under experimental conditions that mimic the physiological milieu?

i) Kinetics and shear-dependence of vWF self-association

We wish to study vWF self-association using SPR[4] since it is not possible to perform such studies in mixed systems with multiple proteins using the light, neutron or X-ray scattering methods of Aim 1. Further, experiments in the SPR apparatus can be designed to mimic the *in vivo* geometry under vascular injury conditions, where the denuded endothelium bearing collagen binds to the A3 domain of vWF, and this first step is followed by further recruitment or self-association of vWF (41).

During this grant, we will employ Surface plasmon resonance (SPR) -based biosensors from Biacore to quantify the kinetics and shear-dependence of vWF self-association. The specific model that the PI will use is a Biacore-X instrument that is available to the PI at a shared user facility at the university. The PI is also part of a pending instrumentation grant from the NIH for the purchase of a Biacore-3000 instrument.

In the Biacore-X, the flow cell has a height of 50 microns (μm) and a width of 0.5mm. Fluid can be flown in this instrument from 1-100 μL/min. The average fluid velocity under these conditions varies from 0.067-6.7cm/s and the wall shear rate varies from 80-8000/s. Thus, in principle, the Biacore instrument could be used to investigate the effects of shear on receptor-ligand binding over both the venular and arterial shear regime. However, in reality, in this device there is a 100nm hydrogel that extends from the sensor chip surface, so that the immobilized receptor experiences less shear than the analyte. Also, practical issues like mass transport limitations and dispersion effects at low flow rates probably require us to move to higher flow rates, above ~7μL/min, for quantitative experiments. These features may limit the range of shear rates in our runs from 500-8000/s.

In our experiments, either collagen or an anti-vWF monoclonal antibody will be immobilized on the surface of a CM5 sensor-chip installed on the Biacore-X. In the first set of runs, purified vWF at varying concentrations will be flown over this surface over a range of flow rates and the rate of vWF self-association will be measured using SPR. In these experiments, we will standardize the experimental system and determine if the self-association of vWF is shear-dependent. Specifically, the binding of platelets via GpIb-vWF interactions has been reported to occur only above a minimum shear rate of ~140/s (44). At lower shear rates, the translocation of platelets on vWF coated substrates ceases. In these experiments, we will also determine if vWF self-association only occurs above a threshold shear rate and we will estimate the forward and on-rates of vWF self-association over a range of shear rates. Once binding on the Biacore substrate has reached saturation (or a high level), elution of the sensor-chip will be performed by flowing buffer that does not contain vWF. These runs will allow determination of the off-rates of vWF-vWF interactions over a range of shear rates. The studies will shed light on the physiological significance of our observation. In some runs, the pH of the elution buffer will be changed so that we may determine if the self-association is pH dependent. In addition, runs will be performed where the buffer composition is changed to either include or exclude divalent salts like Calcium, Magnesium and Manganese to determine the salt requirements for this self-association. If these studies reveal a particular pH or salt composition where self-association is not observed, besides providing novel mechanistic insight, this information would be useful in planning SANS/SAXS runs under shear. By using these specific pH or salt concentrations for the shear experiments, we can study the structure of vWF under shear in the absence of vWF self-association. For all experiments, mathematical analysis on the kinetics of vWF self-association will be performed using the Biacore software and other models that are published in literature (67, 68).

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[4] The biacore SPR instrument consists of a sensor-surface or chip made of glass with a thin conducting film of gold at one surface. Standard chemistry can be used to attach antibodies or other proteins/receptors of interest onto this sensor-surface. An aqueous sample is flown over this surface. Light is reflected at the interface between the two media (glass and flowing media) of different refractive index at a given angle and this phenomenon is called SPR. If the flowing media has a ligand (e.g. vWF) in suspension that recognizes the surface-immobilized receptor (e.g. collagen), there is a change in the intensity of reflected light at any given angle and this is accompanied by a change in the angle where minimum amount of light is reflected. This change in angle of minimum reflected light intensity is proportional to the molecular weight of the ligand bound onto the substrate and the concentration of the bound ligand. Changes in this angle of reflected light due to SPR is thus a measure of receptor-ligand binding kinetics.

We note here that the Biacore instrument offers an elegant system that can be used to address many interesting questions on the mechanism of vWF self-association. This can be achieved by engaging a collaborator who can provide various vWF constructs that either include or lack specific functional domains. This will provide information on the mechanism of vWF self-association. Such studies may be undertaken in our laboratory in the future.

ii) Measurement of vWF-self association in the presence of other blood proteins

In the next set of experiments, we will perform experiments in mixed systems where purified vWF is mixed with either fibrinogen and/or albumin and flown over the sensor surface. For these runs, the protein mixture will be allowed to flow over the substrate bearing immobilized collagen/anti-vWF antibody for a fixed amount of time. During this fixed time, it is expected that the response units measured in the SPR device will be artificially high since both specific and non-specific interactions of blood proteins with take place with the sensor surface. Following this time interval, protein elution will be performed using buffers that lack vWF as described in the previous section. The rate of vWF unbinding from the sensor chip will be monitored and this will be used to estimate the amount of vWF that was specifically bound to the sensor surface prior to elution.

In some experiments, the concentration of fibrinogen and albumin will be varied and the binding/unbinding kinetics will be quantified. Modeling of this data using simple kinetic models will allow us to determine if vWF binding to the sensor surface is blocked by these proteins and if this blocking is specific or non-specific in nature. If the blocking is specific, we anticipate further analysis of the data to determine if the blocking takes place in a i) competitive, ii) non-competitive, or iii) un-competitive fashion. Similarly, if we observe that vWF self-association is augmented in the presence of plasma proteins, we will be interested in determining the degree to which the binding is cooperative. In some experiments the bound substance from the biosensor substrate may be eluted, and mass spectroscopy and/or HPLC analysis of the eluted sample may be performed in order to determine if other proteins are also coupled to vWF during the self-association process. Such analysis can also be used to confirm that the signal observed in the SPR experiments is indeed due to the vWF self-association process.

Our ability to detect vWF self-association in a physiologically relevant milieu is based on the assumption that the rate at which vWF aggregates breakup is slow, i.e. the off-rate of vWF-vWF “bonds” is low. We have made this assumption since we have observed using light scattering that vWF aggregates remain stable for several hours even after stoppage of shear, though these aggregates do breakup over a period of days (data not shown). If the dissociation rate of the self-associated vWF is shear dependent, we may have to design alternative protocols where the Biacore-X flow rate during the wash-step is decreased.

Depending on the outcome of these runs with fibrinogen and albumin, additional runs may be planned to more closely mimic the exact conditions observed *in vivo* by introducing blood plasma. It is however noted that these experiments with whole-plasma will be complex to interpret since in addition to vWF self-association, a shear-dependent metalloprotease belonging to the ADAMTS13 family also exists in blood that cleaves human vWF (24, 25). Even though, the simultaneous interpretation of vWF aggregation along with proteolysis will be challenging, these experiments are worthy of pursuit in our opinion. Overall, data from this set of experiments will likely yield a very rich set of information on the rates and mechanics of vWF self-association.

3) (Aim3): Studies using molecular simulations and other theoretical methods: Can we identify unique structural features in biomolecules that make them act as “force-transducers”?

One of the aims of this proposal is to uncover the dynamic molecular mechanisms that govern the conformational changes of vWF. This includes the time course, the sequence of events, and the energetics that guide the conformational changes. While X-ray data is now starting to provide limited 'snap-shots' of the vWF structure, the detailed dynamic mechanism for the conformational change is lacking. In this regard, molecular simulations provide a complementary tool to obtain information that is not yet possible to access using experimental methods.

There are two goals of this aim: First, we wish to complete longer simulations for the I546V gain-of-function point mutations in Fig. 5 to clearly predict the dynamics of conformational changes in the A1 domain of vWF. Depending on the results, additional point mutations that lead to vWD typeIIb disease may also be pursued. This will involve "computational construction" of mutants of the red residues in Fig. 1. Additionally, negative control mutations like Arg 636 ->Ala (blue residues in Fig. 1) may also be studied. This particular negative control residue is a site for snake venom protein botrocetin binding to vWF (31).

Second, we are interested in introducing a uniform shear force in our simulation of the vWF A1 domain similar to those done in experiments in agitated solution, and we are interested in examining resultant changes in the molecular structure. For such work, we will plan to perform a constant-angular momentum simulation. This can be done either in a micro-canonical (constant-energy constant-volume) ensemble or in a constant temperature-constant pressure ensemble. In a micro-canonical ensemble, the total angular momentum is conserved. In this case, we need to implement a program into CHARMM to set the total angular momentum to a pre-assigned value. For a constant-temperature, constant-pressure ensemble, the angular momentum of the system should be reset periodically to maintain a constant value.

We then plan to perform MD simulations of wild type vWF A1 domain under various values of constant angular momentum. The question to be addressed is whether or not one can induce conformational change. If so: i) what is the structural motif at which this change occurs. And, is this structural motif conserved in the genebank among various species? ii) What is the minimum angular momentum (or centrifugal force) that is required for the conformational change. And, how does this correlate to our fluid dynamic calculations of Fig. 2? iii) Finally, is the conformation change induced by the vWD type IIb gain-of-function mutation similar in nature to the protein structural change upon application of fluid shear?

Depending on the answers to the above questions, we will determine if additional computations can be performed to identify specific protein motifs that may be susceptible to applied force.

4) About the collaborators:

Drs. _____ (Associate Professor of _____, _____) and _____ (Assistant Professor, _____, _____ and _____, _____) and _____ (_____,_____) are listed as consultants on this grant. These investigators will participate in Aim 1 and 3 of this project. _____ is a Physical Chemist with over 90 publications. _____ has used neutron and X-ray scattering in the past and is on the _____. _____ is also an experienced scientist with over 50 peer-reviewed publications. _____ is a member of the _____ development team. Dr. _____ is a senior scientist and a recognized expert in the area of small-angle scattering (SAS). _____ is a pioneer in this area and has taken a major role in the construction of the _____ SANS instrument at _____. _____ is considered to be the best SANS instrument in the nation.

5) Possible problems and alternative strategies

No problems are anticipated with the training program since graduate-level courses are offered each semester and the PI has strong collaborators who will help with the unstructured aspects of this program.

We do not anticipate any major problem with the experiments in Aim1 and Aim 2. In Aim 1, we are confident in our ability to obtain access to the SAXS instrument at the _____ Laboratories since we already have the SANS and light scattering data to justify our needs. One issue that is open to debate is our ability to detect conformation changes in vWF upon application of shear or to determine that vWF aligns in the direction of flow. There is no way to determine this *a priori* without performing the real experiment. Should problems occur in the Biacore experiments (Aim 2), the PI has a current collaborator, _____, who can be approached for help. _____is a recent graduate from our department who works at _____ as an application scientist. The PI served on _____'s thesis committee while he was at _____.

In our opinion, Aim 3 will be the most intellectually challenging aim of this project. The variety of biophysical modeling strategies is large and their interpretation is often complex. Regardless, we believe that it is important to pursue this goal since it will give us fundamental understanding on the mechanics regulating vWF structure and function. This is critical for the design of future experiments in our laboratory.

This grant does not address the contribution of vWF self-association to thrombosis *in vitro* and *in vivo*. While the PI is aware of this important question, we have excluded it from the current proposal since methods to address this question already exist in our laboratory and the spirit of the K02 (as we understand it) is to support the training of the PI in new methods that are currently beyond his grasp.

The experiments and simulations planned here are reasonable and interesting, in our opinion. However, additional funds, besides the PI's salary provided by the K02 grant, will be required to accomplish these goals. While the existing _____ provides some of the resources for the project and the current K02 would provide valuable time, an additional grant may have to be written for supplies, travel and other support.

6) Timeline

The table below illustrates the timeline for this project with respect to the research plan. The course work including the course on ethical conduct in research will be taken in the first 2.5 years.

Aims	***Year 1***	***Year 2***	***Year 3***	***Year 4***	***Year 5***
1 SAXS/ SANS/ Light	←Write SAXS proposal→		←Implement SAXS run →		
	←Complete SANS analysis→				
2 SPR	←Develop knowledge in rheo-optics→		←Build rheo-optics and math. tools for study of biomolecules under fluid flow→		
3 Molecular Simln.	←Learn SPR→	← Implement SPR runs →			
	←Learn molecular simulation→		←Determine if physiological forces are sufficient to cause biomolecule structural changes? →		

7) Gender, Minority Inclusion and Safety Protocols

The proposed project aims at examining the behavior of blood cells and proteins obtained from healthy human adult volunteers (both male and female, and no minority exclusions). Much of the blood for the current work will come from the _____. For these samples, besides information on blood group, HIV and Hepatitis status, no information is provided.

Some experiments with vWF may also require blood draw in our laboratory. For this donors will be selected from a pool of non-smoking healthy adult volunteers at the _____ campus. This group will consist of both male and female volunteers of age 18-45 years. Typically, there will be a 1:1 ratio of male to female volunteers. The experimental protocols proposed in this study and the donor consent forms have been scrutinized and approved by the _____ institutional review board for human subjects. The number of volunteers who have donated in our laboratory over the last two years is large and diverse with regard to gender and ethnic background. The current list of volunteers exceeds 40 persons. The PI is an experienced phlebotomist.

In addition, extreme care is taken while handling blood and other biohazardous substances in our laboratory. The _____ laboratory located in the Department of _____ is an approved BL-2 Biosafety facility. All personnel conducting research in this facility are trained by the Principle Investigator in accordance with standard operating procedures set up in compliance with the OSHA Bloodborne Pathogens Standard. This includes provisions by the PI to comply with the standards outlined by the _____ Office of _____.

E. Human Subjects

Human subjects will be used in this work as a source of fresh blood cells in some experiments. The source of blood will be from healthy volunteers who will be reimbursed for their donation. The experimental protocols proposed in this study and the donor consent forms have been scrutinized and approved by the _____ institutional review board for human subjects (see attached documentation).

1. Normal young (18-45 years of age), healthy adult donors will be used regardless of sex, and ethnic background.
2. Small quantities (20-30cc) of venipuncture blood obtained in heparinized syringes by trained phlebotomists in the laboratory will be used for the proposed study. Typically, the PI who is an experienced phlebotomist will conduct these blood draws.
3. Volunteers are recruited from researchers and support staff in the Dept. of _____, and from student volunteers at the _____ campus. All donors are required to sign an IRB approved consent form.
4. We use sterilized needles, and the individual is in a sitting position for the draw. Pressure is applied to the venipuncture after the draw. The discomfort is minimal.
5. The risk of drawing small quantities of blood is negligible. Further, records regarding any tests performed with individual blood are kept confidential.
6. In many years of research, I have only observed a few individuals become dizzy and a even smaller percentage form hematomas. Our studies only benefit the volunteer in that they contribute to knowledge gained in understanding the vascular functions in adults.

F. Vertebrate Animals

The proposed project does not involve vertebrate animals.

G. Literature Cited

H. Consortium/Contractual Arrangements

Not applicable.

7. Checklist

8. Appendix