Signal Amplification by Pretargeting with Bispecific Antibody for Highly Sensitive *in vitro* Immunofluorescence Imaging

THESIS PRESENTED

BY

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TO

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Abstract:

Monoclonal antibodies have been widely used in biological experiments and clinical therapeutics for sometime, however, use of directly labeled antibodies with fluorophores or therapeutics has been limited by nonspecific accumulation of antibody-drug-conjugates leading to low target to background ratios. Rhodamine B isothiocyanate (RITC) is widely used as a fluorescent tag in a variety of biological experiments. However, its major shortcoming is its self-quenching properties when high specific activity reagents are needed.

The aim of this study is to develop a novel method to increase the fluorescent activity and decrease self-quenching. To increase the specific fluorescent activity of the signal molecules, polymers are loaded with RITC and to prevent quenching, the fluorophores are spaced with Diethylene Triamine Pentaacetic Acid (DTPA) molecules. Targeting is achieved by using the pretargeting technology of bispecific antibody (BsAb) that binds specifically to a target antigen such as myosin as well as captures the polymers loaded with rhodamine and DTPA due to the combination of anti-myosin and anti-DTPA in the form of the bispecific antibody. The signal amplification was demonstrated in canine cardiac myosin coated microscope slides, frozen murine heart sections, cells in culture by epifluorescent microscopy and flow cytometry. In the frozen murine heart tissue sections, an eight-fold increase in fluorescent intensity was achieved relative to the same anti-myosin antibody modified directly with RITC or using a secondary antibody modified with RITC or TRITC.

Successful completion of the above mentioned studies confirm that decreasing of self-quenching and signal amplification can be achieved at the same time by targeting with rhodamine-modified carriers after pretargeting with bispecific antibody.
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List of Acronyms

BsAb: Bispecific Antibody
BSA: Bovine Serum Albumin
PBS: Phosphate buffered saline
PBS-T: Phosphate buffered saline-TWEEN 20
PL: Poly-L-Lysine polymer
DMSO: Dimethyl sulfoxide
RITC: Rhodamine B isothiocyanate
TRITC: Tetramethylrhodamine-5- (and 6)-isothiocyanate
DTPA: Diethylentriamine pentaacetae Acid
DSR-PL: DTPA-Succinyl-RITC-Poly-L-Lysine polymer
HRP: Horseradish Peroxidase
DHM: Dog Heart Myosin
GAM: Goat-anti-Mouse
H9C2: Rat embryonic cardiomyocyte H9C2 cell line
MAb: Monoclonal antibody
OD: Optical density
FACS: Fluorescence-activated cell sorting
HPLC: High-Performance Liquid Chromatography
SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
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1. Introduction:

1.1 Statement of the problem:

Rhodamine B isothiocyanate (RITC) (Fig. 1) is widely used as a fluorescent tag in a variety of biological experiments. However, it has an inherent limitation of self-quenching when high specific activity reagents are generated to increase the signal. The purpose of this study is to develop a novel method to diminish the quenching problem and enhance the sensitivity by signal amplification using RITC conjugated on poly-L-lysine (Fig. 2) with spacer molecules such as DTPA (Fig. 3) that will prevent quenching of rhodamine molecules in close proximity. To enable the specific targeting of these RITC-loaded polymers, the bispecific antibody technology is used. This technology utilizes an antibody that binds a specific antigen, in our case, myosin and is covalently linked to an antibody specific for a capture antigen, the DTPA that is co-loaded on the PL polymers with RITC. To reduce non-specific interaction of cationic poly-lysine polymers with anionic cells surfaces and ground substances, the polymers are also succinylated to render these polymers negatively charged. This combination of pretargeting with bispecific antibody (Fig. 4) and targeting with the DTPA-succinyl-rhodamine B isothiocyanate-poly-lysine (DSR-PL) (Fig. 5) will be tested in vitro on H9C2 rat embryonic myocardioocyte and frozen murine cardiac sections to demonstrate enhanced sensitivity due to signal amplification at the same time decrease background non-specific activity. In addition, these targeting polymers may be modified to carry radioisotopes or therapeutic agents for diagnosis or therapy. When radioisotopes are used, in vivo molecular imaging will ensue. When chemotherapeutic agents are used, drug loaded pro-drug polymers will be generated for greater target specificity and lower background activity. In vitro application of this approach will provide higher sensitivity biomarker identification in cardiovascular diseases and tumors classification. In vivo application
will provide higher sensitivity identification in any biomarkers including those used in cardiovascular injury and tumor imaging, whereas therapeutic imaging will provide targeted delivery of polymer-pro-drug conjugates to various tumors when pretargeted with tumor biomarker specific bispecific antibody. The current study will be limited to in vitro demonstration of target specific signal amplification with purified canine cardiac myosin, H9C2 cell cultures and frozen murine heart sections. The in vivo and therapeutic applications are outside the scope of this Master of Science thesis report.

Figure 1: chemical structure of RITC
Figure 2: chemical structure of Poly-L-Lysine
Figure 3: chemical structure of DTPA

From: http://www.sigmaaldrich.com/united-states.html

Figure 4: Schematic representation of the synthesis of BsAb
Sources: http://www.piercenet.com/browse.cfm?fldID=02040121
Fahy et al. Nucleic Acids Research. 1993, Vol. 21, No. 8 1819-1826

Figure 5: Schematic representation of the reaction between DTPA, RITC mixture with Poly-L-lysine
1.2 Review of Literature:

1.2.1 Myosin as a potential target:

Myosin containing myofilaments belongs to a family of ATP-dependent motor proteins that play an important role in muscle contraction in association with actin. This process of muscle contraction is observed in a wide range of eukaryotic motility. (1) All eukaryotic cells contain myosin but as isoforms, and its structure and function are conserved in evolutionary development. Amoeba actin can bond with rabbit muscle myosin II to produce functional myofilaments. (2) When heart diseases, such as acute myocardial infarction occur, permeability of the myocardial membrane increases, resulting the exposure of myofilaments. (Fig. 6) Therefore, cardiac myosin may be chosen as a marker of myocardial cell death (Khaw et al) due to myosin exposure to the extracellular environment. This was demonstrated by targeting myosin with radiolabeled anti-myosin antibody. Since myosin is a major intracellular, insoluble filamentous protein that has a molecular weight of 500 kDa, it will not be washed away from the intracellular location but remain in situ for an extended period of time relative to other soluble intracellular molecules in the damaged myocardium. Myosin extracted from the infarcted myocardium that is up to 30 days after infarction maintained in near-normal concentrations. Furthermore, cardiac myosin has somewhat unique structural and antigenic features that are similar but not identical to skeletal and smooth muscle myosin. (3)-(10)
1.2.2 Antibody (anti-myosin) mediated targeting:

Myosin is exposed to extracellular environment following irreversible myocardial injury. This exposure may also be achieved experimentally by reagents that induce membrane permeabilization (90% pre-cooled methanol). It was demonstrated that polyclonal anti-myosin radiolabeled with I-125 localized specifically in experimental acute myocardial infarction (Khaw et al. in 1976). The mechanism of localization involved exposure of myosin following sarcolemma disruption as a sign of irreversible myocardial damage. Anti-myosin has since been used for imaging of the myocarditis, acute myocardial infarction, heart transplant rejection and chemotherapeutic drug related cardiotoxicity. Anti-myosin, as Myoscint was approved by the FDA for clinical use. Unfortunately, the company Centocor did not continue commercialization of this clinical reagent.

Fluorescent-labeled antibodies have been used to detect, localize and delineate intracellular structure in vitro. Fluorescent-labeled anti-myosin antibody has been used to delineate the myocardial structure. Therefore, we chose anti-myosin antibody to demonstrate that signal amplification may be achieved using the pretargeting and targeting approaches with bispecific antibody and polymer-fluorophores conjugates.

1.2.3 Rationale for developing bispecific antibody technology for imaging of irreversible myocardial injury:

Although monoclonal antibodies have been widely used in experimental and clinical diagnosis and therapy, the use of directly labeled antibodies has been limited by low specific activity and high background activity leading to low target to background ratio. In order to overcome this problem, bispecific antibodies were developed to decrease the nonspecific
background activity in imaging.(13) Bispecific antibodies are capable of binding to two distinct antigens simultaneously as compared to the conventional mono-specific antibody that only targets one antigen at a time. BsAb has two different antibody moieties joined together as one macromolecular unit. Nisonoff and Rivers originally proposed the concept of conjugating hybrid reagents with dual specificity. The application of bsAb for biological research has advanced substantially over the past two decades to encompass applications in immunoassays, immunotherapy, radioimmunodiagnosis and radioimmunotherapy.(14) Biologically active agents can be selectively delivered by bsAb to the surface of pretargeted cells that could lead to internalization of the drug for enhanced efficacy.(15)

1.2.4 Pretargeting with anti-myosin-anti-DTPA bsAb:

Pretargeting is a method to separate the targeting unit from subsequent administration of therapeutic or imaging units. It is different from the traditional targeting method called “Antibody-Drug-Conjugates” or ADCs. ADCs are made of direct conjugation of the therapeutic units on antibodies as single units. In the pretargeting approach, bsAb is administrated at first and is allowed to localize and aggregate in the target tissues. Then, the signal or therapeutic drug loaded mono- or di-valent haptens or polymers are delivered as separate entities. This technique greatly improves target to background ratio by reducing non-target normal tissue activities.(16)(17)

This thesis reporting pretargeting with anti-myosin-anti-DTPA-Bispecific-Antibody (AM-AD bsAb) followed by DTPA-succinyl-rhodamine-poly-L-lysine polymers (DSR-PL) for immunofluorescent imaging of myosin in cardiomyocytes or frozen murine myocardial sections, enabled demonstration of higher signal and sensitivity due to amplification of the fluorescent signal by reducing the quenching problem that is encountered in multi-Rhodamine fluorophore
conjugated reagents. This pretargeting approach enabled higher signal amplification and sensitivity in *in vitro* immunofluorescence microscopy and flow cytometry.

**1.2.5 Self-quenching mechanism:**

Fluorophore emission can be impacted by reaction with self or other fluorescent and/or non-fluorescent molecules. These interactions can quench the excited fluorophore. The usage of fluorescence quenching is widely applied to determine the activation status of protein and identify gene expression, since the quenchers can be added or removed in response to the biological events. Fluorophore self-quenching occurs very common in simple dye solution; it was observed that fluorescence of fluorescein, Magdala red, or eosin increased with dilution of dye solutions (Walter 1888). It was proposed that the quenching was resulted from “aggregation” and the fluorescence increase when aggregates dissociates into monomers. However, the mechanisms of fluorophore self-quenching are still not entirely understood. The mechanism is a combination of Fluorescence Resonance Energy Transfer (FRET), energy transfer to non-fluorescent dimers as in contact quenching, collisional quenching interaction between dye monomers, and dimerization of the dye (Fig. 7). (18)(19)

![Diagram of the types of fluorescence quenching](image)

*Figure 7: Diagram of the types of fluorescence quenching (18)*
1.3 Specific Aims:

The overall objective of this project is to develop a novel method to increase the fluorescent signal and diminish the self-quenching problem of rhodamine B usually observed when the concentration of rhodamine B is increased on the targeting molecules such as antibodies and to improve specific targeting by combining the fluorophore conjugated carriers and the bispecific antibody technology. The following specific aims will be undertaken to demonstrate the overall objective.

**Aim 1:** To develop a novel method to diminish the self-quenching problem of Rhodamine B isothiocyanate (RITC) and enhance the sensitivity and signal amplification of *in vitro* immunofluorescent-detection.

**Aim 2:** To demonstrate that Rhodamine B isothiocyanate (RITC) and anhydride of DTPA can be used to prepare polymers that contain rhodamine B spaced with DTPA to reduce quenching. Quenching occurs when molecules of rhodamine B are closely packed on carriers. Different molar excess of DTPA and RITC will be used to demonstrate the effect of spacing on the fluorescent intensity of these polymers.

**Aim 3:** Compared fluorescent intensities between the pretargeting and targeting with bsAb and polymer reagents to the anti-myosin-RITC that targets the myofilaments directly, anti-myosin, and anti-DTPA, followed by rhodamine labeled secondary antibody.

**Aim 4:** The application of directly fluorophore labeled antibodies has been limited by low specific activity and the potential for denaturation of the antibodies when multiple fluorophores are attached covalent to antibodies. Signal amplification will be demonstrated using pretargeting with bispecific antibody and targeting with multiple rhodamine conjugated DTPA-succinyl polylysine polymers (DSR-PL) with canine heart myosin fixed on glass slides.
The signal amplification will be compared to various control immunofluorescent staining reagents.

**Aim 5:** To demonstrate targeted signal amplification in *in vitro* immunofluorescence imaging of frozen murine heart sections after pretargeting with bispecific anti-myosin-anti-DTPA antibody and targeting with DSR-PL. The same controls will be used to demonstrate signal amplification with our technique.

**Aim 6:** Demonstrate signal amplification by flow cytometry of permeabilized H9C2 rat embryonic cardiocytes after pretargeting with anti-myosin-anti-DTPA bispecific antibody and targeting with DSR-PL. The same controls as described above will be included.

**1.4 To achieve the specific aims the following studies will be undertaken:**

- ELISA for assesses the binding of different molar of DTPA and RITC to PL.
- Preparation and characterization of different molar excess of DSR-PL by ELISA, Sephadex-G25 size exclusion chromatography.
- HPLC used to differentiate and separate different DSR-PL polymers.
- In-house production and characterization of anti-DTPA and anti-myosin by Protein G Column purification.
- Preparation and characterization of anti-myosin-RITC, GAM-RITC by ELISA.
- Epifluorescent microscopy to demonstrate the fluorescence effect of the RITC conjugated anti-myosin and control antibodies.
- Preparation and characterization of bispecific antibody complex (anti-DTPA - anti-myosin) by ELISA and SDS-PAGE.
• *In vitro* targeting of Dog Heart Myosin (DHM) after pretargeting with bsAb complex and targeting with 20X and 30 X DTPA-succinyl-rhodamine-PL by epifluorescence microscopy.

• Demonstration of *in vitro* targeting of frozen murine heart sections (three sets) after pretargeting with bsAb complex and targeting with 20X and 30X DTPA-succinyl-rhodamine-PL by epifluorescence microscopy.

• Demonstration of enhanced *in vitro* targeting in permeabilized H9C2 rat embryonic cardiocytes after pretargeting with bsAb complex and targeting with 20X and 30 X DTPA-succinyl-rhodamine-PL by flow cytometry.
2. Materials and Methods:

2.1 Production of Ascites Fluid:

Anti-DTPA and anti-Myosin monoclonal antibodies were produced by mouse ascites method. This antibody production method involves two steps: priming of the peritoneal cavity and inoculation of hybridomas. The priming was achieved by injection of 0.2 ml of pristine to induce granulomatous reactions in intraperitoneum. Fourteen days after priming, 0.15 ml of 2G42D7 and 2C31E11C7 hybridoma cells (1.5 X 10^6) was inoculated into the peritoneal cavity of mice. After hybridoma inoculation, animals were carefully monitored daily. Abdominal paracentesis was carried out three times when the abdominal distension was evident. The abdominal area was sterilized before the ascitic fluid was collected with a 22-gauge needle. The ascitic fluid was then processed for purification of monoclonal antibodies.

2.2 Processing of the Ascites Fluid:

The ascetic fluid was kept at room temperature for about 30 minutes after harvest. Then, test tubes were centrifuged for 15 minutes at 3000 rpm to get rid of the red blood cells. The supernatant was centrifuged for 15 minutes at 20,000 rpm to remove fatty material. After that, the ascites fluid was treated with 0.1N HCl (pH 4.9-5.1) and incubated at 4°C for one hour to precipitate fibrinogen. After incubation, the ascetic fluid was again centrifuged for 15 minutes at 20,000 rpm to remove fibrinogen. The ascetic fluid was finally treated with 0.1M NaOH to raise the pH to 8.0.(20)

2.3 Affinity Purification of Monoclonal Antibodies:

The processed ascetic fluid containing monoclonal antibody was dialyzed overnight against binding buffer (20mM Na_2HPO_4, 0.15 M NaCl, pH 8.0). The dialyzed ascetic fluid was mixed with equal amount of binding buffer and was then applied to Protein-G column affinity
chromatography (GenScript). The fall-through was collected at a flow rate of 1ml/min. The column was washed with binding buffer with a flow rate of about 2 ml/min until the OD$_{280}$ of the effluent was zero. The antibodies were then eluted with elution buffer at the flow rate of 1 ml/min (0.1M Glycin, pH 2.5). The eluted antibody was collected in 1ml fractions in sterilized tubes pre-filled with 100 µl of neutralizing buffer (1M Tris-HCl, pH 8.5). After elution and neutralization, the antibody was dialyzed overnight against 0.1M PBS, pH 7.4 and was characterized for its immunoreactivity (ELISA) and purity by SDS polyacrylamide gel electrophoresis at 6.0% acrylamide concentration. The concentration of purified antibody was determined by UV spectroscopy at 280 nm optical density reading (SPECTRONIC, GENESYS5). The purified antibody was stored at -20ºC for future use.(21)

2.4 Characterization of Monoclonal Antibodies (SDS-PAGE)

The purity of the monoclonal antibodies (anti-DTPA and anti-Myosin) was characterized by SDS-PAGE (BIO-RAD Mini-PROTEAN Tetra cell kit). The gels were made by 6% resolving gel and 4% stacking gel (Acrylamide/Bis). Aliquots of 10 µg protein and bromophenol blue (30 µl) were prepared in sample buffer. The samples were pre-heated for about 5-10 minutes before loading on the gel. The MOPS-SDS running buffer (100mM 3-(N-morpholino) propane sulfonic acid (MOPS), 6.93mM Sodium dodecyl sulfate (SDS), 100mM Tris Base, 2.05mM EDTA) was used for running the gel (kept in the dark until use). The electrophoresis was undertaken at 200 V and a run time of about 30 minutes. After the electrophoresis, the gel was removed from the gel cassette and was further processed for Coomassie Blue Staining. The gel was rinsed with three changes of deionized water and was kept in 0.0025% coomassie blue (staining solution) for about three hours. The gel was then de-stained with three changes of de-
staining solution (40 % methanol, 10 % glacial acetic acid). After de-staining, the gel was rehydrated with deionized water.

2.5 Preparation of Bispecific antibody complex - Crosslinking of anti-DTPA to anti-Myosin:

Intact anti-DTPA antibody (1.5mg/ml) was dialyzed overnight in 50mm NaCl/1mM EDTA solution (pH 8.0). The dialyzed anti-DTPA antibody was then modified with 100X molar excess of 2-iminothiolane that was dissolved in 200 µl of anhydrous dimethyl sulfoxide (DMSO) (Sigma). Intact anti-Myosin antibody was dialyzed overnight in 0.1 M PBS (pH 7.4). The dialyzed anti-Myosin antibody was then modified with 100X molar excess of Bromoacetic acid N-hydroxy succinimide ester (Sigma). Both the modification reactions were carried out at 4°C for 6 hours followed by their separation using column centrifugation with Sephadex G-10 column (10 ml PD-10 columns). 0.1M PBS (pH 7.4) was used as the elution buffer. Trinitro Benzene Sulfonic (TNBS) assay was performed to access the extent of modification of antibodies. The equal-molar concentrations of modified antibodies were then incubated at 4°C overnight to conjugate anti-DTPA antibody to anti-Myosin antibody via thioether bonds. The free monoclonal antibodies (150kDa) were then separated from the bispecific antibody complex (300kDa) by HPLC size exclusion chromatography using a Zorbax-GF 250 column (Agilent technologies). (Panwar R. Ph.D. Thesis)

2.6 Isolation of Bispecific antibody complex (anti-DTPA X anti-Myosin):

The free monoclonal antibodies (150kDa) were separated from the bispecific antibody complex (300kDa) by HPLC size exclusion chromatography using a Zorbax-GF 250 column (Agilent technologies) (9.4 x 250 mm) (size exclusion limits=400,000 Daltons to 4,000 Daltons). Zorbax-GF 250 size exclusion column was first washed with 0.2 M sodium phosphate buffer at pH 7.0. It was then calibrated by using different standards such as Bovine serum albumin (BSA),
anti-DTPA and IgG. The mixture of monoclonal antibodies and bispecific antibody was dialyzed in 0.2 M sodium phosphate buffer (pH 7.0). An aliquot of 350 µl of sample was applied to the column and 250 µl aliquot fractions were collected. The flow rate of 500 µl/min at a pressure of 350psi was used. Optical density (OD) at 214nm was read to determine the elution profile. (22)

2.7 SDS-PAGE for Identification of Bispecific Antibody

The Bispecific antibody complex was identified by SDS-PAGE (BIO-RAD Mini-PROTEAN Tetra cell kit). The gels were composed by 6 % Acrylamide/Bis casted resolving gel and 4% Acrylamide/Bis stacking gel. After the gel has polymerized (30 mins), the preheated samples containing 10 µg proteins and sample buffer containing bromophenol blue tracking dye (30 µl) were loaded to the gel. The electrophoresis was conducted at 200 V and a run time of about 30 minutes. After electrophoresis, the gel was processed for Silver Staining. The gel was rinsed with three changes of deionized water for 30 minutes. The gel was then fixed for 10 minutes in the fixing solution (10 % glacial acetic acid, 50 % ethanol) followed by washing and rinsing solution (50 % ethanol) for 5 minutes. After the rinsing step, the gel was sensitized with 0.02% Sodium Thiosulfate for 2 minutes. The gel was washed with two changes of deionized water. The gel was then stained for 20 minutes with 0.02% Silver Nitrate solution in the 4°C refrigerator. Following the incubation at 4°C, the gel was washed with two changes of deionized water for 2 minutes. Then the gel was kept in the developing solution (10g Sodium carbonate, 200µl of 37% v/v Formaldehyde and 20ml of Sensitizer) for about 1-3 minutes. The stop solution (1% Acetic acid) was applied to stop the color develop. The gel was then transferred to a wet filter paper followed by overlaying with a plastic sheet. The prepared gel was then transferred for drying (Bio-Rad gel dryer, model # 583). Anti-Myosin and anti-DTPA monoclonal antibodies were used as monomeric controls.
2.8 ELISA for Bispecific antibody complex (Anti-DTPA and Anti-myosin):

2.8.1 ELISA for Anti-DTPA:

Anti-DTPA antibody activity after modification with 2-iminithiolane was evaluated by ELISA. A 96-well micro titer plate (BD Biosciences) was used for the ELISA. 100µl of DTPA-BSA (1µg/ml) was added to each well of the micro-titer plate and covered with parafilm (PECHINEY PLASTIC PACKING), followed by incubation at 37°C water bath for one hour. After incubation, the plate was washed five times with 0.1M PBS-T. The wells were then blocked with 200µl/well of 3% Bovine Serum Albumin (BSA) for one hour at 37°C. Following incubation the plate was washed five times with 0.1M PBS-T. Then 100µl aliquots of serial dilutions starting with 1 µg/ml of purified monoclonal anti-DTPA antibody as a positive control and purified bsAb were also added to the micro-titer wells. The plate was incubated at 37°C for one hour and washed with 0.1M PBS-T (5X). 50µl of GAM-HRP (1:5000 dilution) was loaded to the plate. The plate was incubated at 37°C for one hour. After incubation, the plate was washed with 0.1M PBS-T (5X). Then, 50µl aliquots of K-Blue substrate (Chromogen) were added to each well. The plate was kept in dark for about 5-10 minutes. The plate was then read at 630 nm and binding data were evaluated by GEN 5.0 software (Biotek Instruments).

2.8.2 ELISA for anti-Myosin:

The immunoreactivity of anti-Myosin antibody modified with N-hydroxy succinimide ester of bromoacetic acid (Sigma) was assessed by ELISA. Aliquots of 100µl/well of Canine (Dog) Heart Myosin (DHM) (1µg/ml) dissolved in 0.3M PBS was added to the wells of a 96-well micro plate (BD Biosciences) and incubated at 37°C for one hour. The plate was then washed with 0.1M PBS-T (5X). After washing, 200µl/well of 3% BSA was added for blocking. Then the 96-well plate was incubated at 37°C for one hour followed by washing with 0.1M PBS-
T (5X). Serial dilutions starting with 100 µl of 1 µg/ml of anti-DTPA-anti-Myosin Bispecific antibody complex were added to the 96-well plate. The same serial dilutions of purified monoclonal anti-Myosin antibody was added as positive control, and the plate was incubated at 37ºC for one hour. The wells were then washed with 0.1 M PBS-T (5X) and 50µl aliquots of GAM-HRP (1:5000 dilution) was added to the plate and incubated in 37ºC for one hour. After incubation, the plate was washed with 0.1M PBS-T (5X). Followed that, 50µl aliquots of K-Blue substrate (Chromogen) were added to each well. The plate was kept in dark for about 5-10 minutes. The plate was then read at 630 nm and binding data were evaluated by GEN 5.0 software (Biotek Instruments).

2.9 Preparation of 20X, 30X DSR-PL:

20 mg of Poly-L-lysine hydro-bromide (PL, MW 13.8 kDa) (Sigma Chemical Co.) was solubilized in 2 ml of 0.1 M Na₂CO₃ at pH 8.7. The mixture of 20 molar excess of anhydride of DTPA (SIGMA-ALDRICH Chemical Co.) and 20 molar excess of RITC (Sigma Chemical Co. FW 536.1) in 0.1 ml of dimethyl sulfoxide (DMSO) was added to the PL solution drop by drop-wise while mixing vigorously using a vortex mixer. The mixture was gently mixed for approximately 5 minutes followed by incubation at room temperature for 2 hours. Then, the Rhodamine conjugated DTPA- PL mixture was subjected to Sephadex G-25 (GE Healthcare Co.) column chromatography (10 cm X 1 cm) to separate the DSR-PL from free RITC and DTPA. The column was pre-calibrated with Blue dextran 2000. Then peak tubes containing the bsAb were pooled and dialyzed overnight against excess (4 L) 0.1M PBS pH 7.4 at 4ºC in the dark. The DSR-PL was then subjected to 100 molar excess of succinic anhydride (Sigma Chemical Co.) for succinylation. The resultant DSR-PL was dialyzed against excess (4 L) 0.1M PBS pH 7.4 at 4ºC in the dark.
2.10 HPLC of DSR-PL polymers

20X, 30X DSR-PL and naked polymers were characterized by HPLC chromatography using Zorbax-GF 250 size exclusion column (9.4 D mm x 250 L mm). An aliquot of 100µl of the sample was applied to the Zorbax GF-250 size exclusion column that was pre-equilibrated and calibrated with proteins of known molecular weights such as BSA. The mobile phase of the column was 0.2M sodium phosphate buffer (pH 7.0). The elution was monitored at OD 220nm, 280 nm and fluorescence detector. The flow rate of the column was optimized to 1ml/min and the pressure of the column was maintained at around 350psi.

2.11 Preparation of Anti-myosin-RITC:

RITC conjugated to anti-myosin was prepared to be used as a control and fluorescent reagent for comparison to the fluorescence of 20X, 30X DSR-PL after pretargeting with bsAb. Anti-myosin was dialyzed in 0.1M PBS. The concentration of the anti-myosin (1mg/ml) was determined by a spectrophotometer (Extinction coefficient for IgG at A(280) = 1.4). RITC is covalently conjugated to primary amines (lysines) on the antibody to form thiourea linkage. An aliquot of 10 molar excess of RITC in 0.1ml of anhydrous DMSO was prepared immediately prior to conjugation. The RITC solution was added to the anti-myosin solution drop by drop in a 15 ml falcon tube while mixing vigorously (vortexing). The falcon tube was covered with aluminum foil. Then the mixture was gently stirred for approximately 5 minutes followed by incubation at room temperature for 2 hours. Then reaction mixture was dialyzed overnight against excess (4 L) 0.1M PBS pH 7.4 at 4ºC in the dark. The immunoreactivity of anti-myosin-RITC was determined by ELISA.(23)

2.12 Preparation of Goat-anti-Mouse-Rhodamine B isothiocyanate (GAM-RITC):
Goat-anti-mouse antibody GAM (CAPPEL) was conjugated with RITC to be used as a control for comparison against the 20X and 30X DSR-PL after pretargeting with bsAb. RITC is covalently conjugated to GAM as described for conjugation to anti-myosin as described above.

2.13 Cell Culture:

Embryonic rat cardiomyocytes (H9C2 cell line) are grown in DMEM supplement (500 ml) with 10% Fetal Clone II (Hyclone 50 ml) and 1% penicillin, streptomycin and fungicidal agent amphotericin (2.5 µg/ml of 5.5 ml) (Sigma Company Co.) at 37 °C in 5% CO₂. For in vitro studies, 40,000 cells/well are grown in 12 well plates until 80% to 90% confluency is obtained. The cells are dissociated by 1X Trypsin-EDTA digestion (Sigma Company Co). The cells are either passed for used in the studies as described below.

2.14 Cell Viability Test (Trypan Blue exclusion test):

Trypan Blue exclusion test will be used to assess cell viability. This dye is excluded by the live cells but not by dead cells, therefore, dead cells accumulate the blue dye and appear blue under optical microscopy. An aliquot of 20µl of cell suspension is added to a 1 ml Eppendorf tube and then an equal volume of 0.5% (w/v) trypan blue is added to the same tube and mixed well gently. Then 10µl of the mixture is loaded on a hemocytometer pre-covered with a cover slide. Dead cells are counted using an inverted microscopy (Olympus ck2; Optical, Inc. Hudson, MA) and view at magnification 100X. Dead cells are stained with blue color and number of viable cells can be calculated.(24)

2.15 Preparation of frozen tissue sections:

The mouse hearts were collected from freshly exsanguinated mice and each heart was placed in a tissue holder that had been filled with the cryo-embedding media O.C.T compound (Tissue-Tek). The tissue holder containing the heart block was immersed in liquid nitrogen until
the entire heart block was frozen completely. Then, the frozen heart was stored at -80°C until used for sectioning. The frozen heart sections prepared using the Microm HM550 (Thermo Scientific, Kalamazoo, MI.). The temperature of the cryotome chamber was set at -20°C. The base of the holder was build up with freezing medium and the frozen heart block was mounted on it and returned to the chamber. Freezing medium was added to the top of the frozen heart block to cover the whole block. The base was allowed to freeze for 5 minutes. The mounted heart was examined for proper mounting, then 10 microns thick sections were cut. The mount angle of the heart was set at position 6. The vacuum pressure of the cryotome was set at 9.5. The mount was directed up and down by using the side scroller. The cut sections were mounted on clean glass slides by using a clean brush method. During transfer the sections onto the glass slide, care was taken to avoid thawing of the tissues. After preparing the slides, the heart tissue sections were dried overnight at room temperature. The frozen mounted sections were could be stored in a sealed slide box at -80°C for future use.

2.16 Immunostaining of the frozen murine heart sections for histology and epifluorescence studies:

The frozen heart tissue section slides were immersed in pre-cooled acetone (-20°C) for 10 minutes to fix the section to the microscope slide. Then the pre-cooled acetone was decanted and the acetone was allowed to evaporate from the tissue sections for 20-30 minutes at room temperature. Each slides was rinsed with 0.1M PBS (5X), 3-5 minutes each wash. Then, the tissue region of the slides was layered with 100 µl of 3% BSA in 0.1M PBS and incubated in a moist chamber at room temperature for 1 hour to block nonspecific binding. The blocking reagent buffer was decanted and the slides were washed with excess 0.1M PBS (5X), 3-5 minutes each wash. Then, 100 µl of an appropriate dilution of primary antibody was added to
the tissue section on the slides and incubated in the moist chamber for one hour at room temperature. The slides were then washed with 0.1M PBS-T (5X), 3-5 minutes each wash followed that washed with 0.1M PBS (5X), 3-5 minutes each wash. After washing, aliquots of 100 μl of appropriately diluted secondary antibody was added to the slides and incubated in the moist chamber for one hour at room temperature. The slides were then washed with 0.1M PBS-T (5X) and 0.1M PBS (5X), 3-5 minutes each wash. Then the tissue slides were dehydrated with a series of alcohol concentrations 70% (two times)/95% (two times)/100% (two times) and kept moisture in the moist chamber and one drop of Fluoromount-G (SouthernBiotech) was added to each tissue slide. Then the cover slide was put on the embedded tissue and sealed with clear nail polishing. After the nail polishing has dried, the mounted slides were stored in -20°C in a tissue slide box for subsequent epifluorescence microscopic examination. (Nikon Eclipse from Dr. Torchilin’s lab)(25) The excitation wavelength of 528-553nm, DM 565nm and BA 600-660nm was set for epifluorescent microscopy examination. Ocular: CFI 10X22M. Objective: Plan 20X 0.40 PH1 DL. Digital micrographs were acquired at an exposure time of 800ms and Gain was set at 4. White Balance was set at 1.013, 1, 1.126. The image size was Image size: 1600 X 1200 pixels Bit Depth: 24 bpp(RGB) Sensor Clear Mode: Continuous Chip Area: Full Chip.

2.17 FACS study:

Cultured H9C2 rat embryonic cardiomyocytes cells were cultured in 12 well plates starting with 40,000 cell/well. After 80-90% confluency, cells were trypsinized and neutralized with DMEM cell culture medium. Then, the cell pellets were suspended and washed in 2ml of 0.1M PBS. An aliquots of 500 μl of 90% pre-cooled methanol (-20°C) was used to permeable the cells for 30 minutes in 4°C. Then the cells were washed two times with 2 ml of 0.1M PBS. After blocking with 100 μl of 3% BSA, the cells were treated with different reagents as follows: 1
µg/ml anti-myosin+10 µg/ml GAM-RITC or GAM-TRITC, 1 µg/ml anti-DTPA+10 µg/ml GAM-RITC or GAM-TRITC, 1 µg/ml bsAb+1 µg/ml 20X or 30X DSR-PL, 1 µg/ml of 20X or 30X DSR-PL alone, and 5 µg/ml, 1 µg/ml of anti-myosin-RITC. The number of cells and the staining of cells in each sample were assessed by flow cytometry (FACS Calibur instrument, BD Biosciences, San Jose, CA) equipped with an argon-ion laser and an optional second red diode laser (source energy, 15 mW; detection time, 500 counts per second). Data were live gated for 10,000 cells each by Forward light scatter (FSC) and Side light scatter (SSC) ($\lambda_{ex}$= 488 nm, $\lambda_{em}$=585/42nm). Cell Quest pro software was used for data acquisition and analyses (BD Biosciences, San Jose, CA).

2.18 Statistics:

Data was expressed as Mean+/−SD. The statistical package of Microsoft Excel 2010 was used for data analysis.
3. Results:

3.1 ELISA for Characterization of BsAb:

ELISA was performed to assess the immunoreactivity of anti-myosin-anti-DTPA BsAb made previously by Dr. Panwar who was a senior member of Dr. Khaw laboratory. DTPA-BSA was used as the antigen for estimating the immunoreactivity of anti-DTPA antibody while dog heart myosin (DHM) was used for testing the immunoreactivity of anti-Myosin antibody. Fig. 8 and 9, show the ELISA data confirming that the immunoreactivities of anti-DTPA and anti-myosin of bispecific antibody were retained relative to the immunoreactivity of the unmodified antibodies used as controls.

**Figure 8:** ELISA for analysis of the immunoreactivity of the anti-DTPA activity of the BsAb (green diamond curve) relative to un-modified anti-DTPA antibody (blue diamond curve)
Compare immunoreactivity between anti-myosin standard and anti-myosin on BsAb

Figure 9: ELISA for analysis of the immunoreactivity of the anti-myosin antibody activity of the BsAb (green diamond curve) relative to un-modified anti-myosin antibody (blue diamond curve)

3.2 SDS-PAGE for BsAb:

SDS-PAGE was performed to determine the purity of the BsAb. Lane #1 is and #2 (Fig.10) represents two different bsAb (anti-DTPA-anti-myosin) samples. Lane #1 sample may contained residual monomeric IgG antibody seen only in the left most #1 lane, whereas only the high molecular weight dimeric species is evident in lane #2. Therefore, the #2 bsAb was used in the following studies.

Figure 10: SDS-PAGE to determine purity of BsAb antibody
3.3 ELISA for immunoreactivity of Goat Anti Mouse-RITC antibody:

Figure 11: Assessment of the immunoreactivity of GAM-RITC by ELISA. (Upper line: standard GAM---blue diamond curve, Lower line: GAM-RITC---green diamond curve)

Fig. 11 shows the ELISA confirming that the immunoreactivity of the GAM-RITC was retained relative to the untreated GAM used as a control.

3.4 Assessment of the immunoreactivity of anti-myosin-RITC antibody:
Figure 12: Comparison of binding of unmodified anti-myosin-2G42D7 (blue diamond curve) and RITC conjugated anti-myosin 2G42D7 (green diamond curve)

Fig. 12 shows the immunoreactivity of the anti-myosin-RITC relative to control anti-myosin antibody. There is no difference in the two curves indicating that the immunoreactivities of these two anti-myosin samples are identical.

3.5 Characterization of 20X, 30X DSR-PL:

3.5.1 Sephadex G-25 size-exclusion chromatography of Blue Dextran, 20X, 30X DSR-PL and free RITC (Figure 13 and Figure 14)

![Figure 13---Sephadex G-25 elution profile of Blue Dextran (0.5ml/fraction)](image)

Fig. 13 shows the elution profile of Blue Dextran on a Sephadex G-25 column (1 x10 cm) to assess the void volume of the column. The void volume of Sephadex G-25 size-exclusion column is approximately 2.5 ml.

![Figure 14---Sephadex G-25 elution profile of 20X, 30X DSR-PL and free RITC](image)

Fig. 14 shows the elution profiles of 30X (blue), 20 X (red) DSR-PL, and free rhodamine (green) determined at OD557nm. Both 30 X and 20X DSR-PL are eluted in the void volume.
indicate that the rhodamine was conjugated to the polymers. No inclusion peak equivalent to free rhodamine is present in the 30X and 20X DSR-PL samples. Therefore, both 30X and 20X DSR-PL are considered to be free of unreacted rhodamine.

3.5.2 Table I: TNBS Assay for DTPA-BSA Modification Rate

<table>
<thead>
<tr>
<th>Sample</th>
<th>dH2O (µl)</th>
<th>0.1 M Sodium Tetraborate</th>
<th>TNBS (2mg/ml)</th>
<th>Incubate</th>
<th>Na2SO3 (1.5mg/ml in 50 ml of 0.2M NaH2PO4)</th>
<th>OD 420nm</th>
<th>Mean OD 420 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>1 0</td>
<td>375 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 0</td>
<td>375 µl</td>
<td>187.5 µl</td>
<td>for 30 min</td>
<td>375 µl</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 0</td>
<td>375 µl</td>
<td>187.5 µl</td>
<td>at 37 °C</td>
<td>375 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>1 3</td>
<td>372 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.204667</td>
<td>0.216</td>
</tr>
<tr>
<td></td>
<td>30 µg/well</td>
<td>2 3</td>
<td>372 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.239667</td>
</tr>
<tr>
<td></td>
<td>10 mg/ml</td>
<td>3 3</td>
<td>372 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.203667</td>
</tr>
<tr>
<td>BSA</td>
<td>1 6</td>
<td>369 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.316667</td>
<td>0.34766666</td>
</tr>
<tr>
<td></td>
<td>60 µg/well</td>
<td>2 6</td>
<td>369 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.396667</td>
</tr>
<tr>
<td></td>
<td>10 mg/ml</td>
<td>3 6</td>
<td>369 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.329667</td>
</tr>
<tr>
<td>BSA</td>
<td>1 9</td>
<td>366 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td></td>
<td>0.51833333</td>
</tr>
<tr>
<td></td>
<td>90 µg/well</td>
<td>2 9</td>
<td>366 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.537667</td>
</tr>
<tr>
<td></td>
<td>10 mg/ml</td>
<td>3 9</td>
<td>366 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.511667</td>
</tr>
<tr>
<td>Modified BSA-DTPA</td>
<td>1 7.2</td>
<td>368 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.060667</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>30 µg/well</td>
<td>2 7.2</td>
<td>368 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.060667</td>
</tr>
<tr>
<td></td>
<td>4.2 mg/ml</td>
<td>3 7.2</td>
<td>368 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.070667</td>
</tr>
<tr>
<td>Modified BSA-DTPA</td>
<td>1 14.3</td>
<td>361 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.134667</td>
<td>0.09766667</td>
</tr>
<tr>
<td></td>
<td>60 µg/well</td>
<td>2 14.3</td>
<td>361 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.051667</td>
</tr>
<tr>
<td></td>
<td>4.2 mg/ml</td>
<td>3 14.3</td>
<td>361 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.106667</td>
</tr>
<tr>
<td>Modified BSA-DTPA</td>
<td>1 21.4</td>
<td>354 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.103667</td>
<td>0.09733333</td>
</tr>
<tr>
<td></td>
<td>90 µg/well</td>
<td>2 21.4</td>
<td>354 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.137667</td>
</tr>
<tr>
<td></td>
<td>4.2 mg/ml</td>
<td>3 21.4</td>
<td>354 µl</td>
<td>187.5 µl</td>
<td>Incubate</td>
<td>375 µl</td>
<td>0.050667</td>
</tr>
</tbody>
</table>

DTPA-modified BSA is used to assess anti-DTPA antibody activity as well as for quantitation of DTPA incorporated in PL. To quantitate the incorporation of DTPA in BSA, the TNBS assay that react with the lysyl residues on BSA is used. Table I shows the TNBS values representing modification of lysyl residues on BSA prepared previously by senior member of Dr. Khaw’s Lab by reaction with anhydride of DTPA (Concentration= 1 mg/ml). Modification of BSA was calculate as \( \frac{(\text{OD} 420 \text{ nm of un-modified BSA} - \text{OD} 420 \text{ nm of modified BSA})}{\text{OD}420 \text{ nm of unmodified BSA}} \times \text{number of lysyl residues in BSA} = \left[ \frac{0.216-0.046}{0.216} \right] \times 90 = \text{46} \). Therefore, there are 46 moles DTPA/mole of BSA.
3.5.3 Biuret Assay for 20X, 30X DSR-PL Concentration

**Table II. Biuret Assay for determine the Concentration of 20X, 30X DSR-PL**

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Concentration (mg/ml)</th>
<th>Stock PL (20 mg/ml)</th>
<th>0.1 M Na2CO3</th>
<th>Biuret Reagent (ml)</th>
<th>Incubate 37°C</th>
<th>OD(550nm)</th>
<th>Mean OD(550nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>25 µl</td>
<td>975 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.015</td>
<td>0.003</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>50 µl</td>
<td>950 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.023</td>
<td>0.027</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>125 µl</td>
<td>875 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.098</td>
<td>0.064</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>250 µl</td>
<td>750 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.139</td>
<td>0.137</td>
</tr>
<tr>
<td>5</td>
<td>7.5</td>
<td>375 µl</td>
<td>625 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.197</td>
<td>0.194</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>500 µl</td>
<td>500 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.263</td>
<td>0.253</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>750 µl</td>
<td>250 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.367</td>
<td>0.368</td>
</tr>
<tr>
<td>20X DSR-PL(1:100) X</td>
<td>10 µl of stock sample</td>
<td>990 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.024</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>30X DSR-PL(1:100) Y</td>
<td>10 µl of stock sample</td>
<td>990 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.032</td>
<td>0.031</td>
<td>0.032</td>
</tr>
<tr>
<td>20X DSR-PL(1:100)background</td>
<td>5.525 µg of RITC</td>
<td>1000 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.018</td>
<td>0.017</td>
<td>0.017</td>
</tr>
<tr>
<td>30X DSR-PL(1:100)background</td>
<td>2.838 µg of RITC</td>
<td>1000 µl</td>
<td>4</td>
<td>30 minutes</td>
<td>0.023</td>
<td>0.027</td>
<td>0.024</td>
</tr>
</tbody>
</table>

**Figure 15: Standard Curve for Poly-L-Lysine (13.8 kDa)**

\[ y = 0.0247x + 0.0069 \]
\[ R^2 = 0.99529 \]

From Table II and Fig. 15, the concentration of 1:100 dilution of 20X, 30X DSR-PL was 0.004 mg/ml calculated from the equation of the straight line \( y=0.0247x+0.0069 \) (\( R^2=0.99529 \)), therefore, the original concentration of the stock 20X, 30X DSR-PL was 0.4 mg/ml.
3.5.4 Absorbance spectrum of Rhodamine B Isothiocyanate (RITC)

As shown in the Fig. 16-A and B, maximum absorbance of RITC was observed at 557nm. This absorbance was used to generate a concentration curve for assessment of the concentration of RITC on the polymers.
Standard Curve for RITC for determination of RITC on Polymers

![Standard Curve for RITC](image)

Figure 17: RITC standard curve at OD₅₅₇nm

Sephadex G-25 gel size exclusion chromatography in Fig. 14 shows that the 30X DSR-PL elution absorbance at 557 nm and that of 20X DSR-PL. Using the equation of the line y=0.0156x-0.0127 (R²=0.9961) (Fig.17), the concentration of RITC on 30X DSR-PL and 20X DSR-PL are calculated to be 552.5 µg/ml and 283.8 µg/ml.

For the 30X DSR-PL, there are 552.5X10⁻⁶/536=1.03X10⁻⁶ moles of RITC and 0.4X10⁻³/13800 = 2.899X10⁻⁸ moles of PL. Therefore, 1 mole of 30X DSR-PL has 1.03X10⁻⁶/2.899X10⁻⁸=36 moles of RITC.

For the 20X DSR-PL, there are 283.8X10⁻⁶/536= 0.528X10⁻⁶ moles of RITC and 0.4X10⁻³/13800 = 2.899X10⁻⁸ moles of PL. Therefore, 1 mole of 20X DSR-PL has 0.528X10⁻⁶/2.899X10⁻⁸=18 moles of RITC.
3.5.5 ELISA for assessment of DTPA in 20X and 30X DSR-PL:

![ELISA graph](image)

Figure 18: ELISA for determination of DTPA concentration by binding of 20X, 30X DSR-PL

Fig. 18 shows the ELISA data for determination of the concentration of DTPA on 20X and 30X DSR-PL. Different dilutions of 100 µl of polymer starting from 0.1 dilutions were added to coat the 96 well plate. After 200 µl of 3% BSA blocking for 1 hour in 37°C incubator, 100 µl of 1µg/ml anti-DTPA was added as primary antibody. After 1 hour incubation in 37°C incubator, 50 µl of the GAM-HRP (1:5000 dilution) was added as secondary antibody. Followed 1 hour incubation in 37°C incubator, 50 µl of K-blue was added and kept in dark for 5 minutes and the stop solution was added and the plate was read at 450nm.

For 30XDSR-PL, 1 µg/ml of DTPA-BSA shows equivalent binding to that of 0.006X0.04mg/ml= 0.24 µg/ml of 30X DSR-PL, which equal to 1.74X10^{-11} moles of PL. Since there are 46 lysine residues are modified in the DTPA-BSA (46 moles of DTPA/mole of BSA), 1 µg/ml of DTPA-BSA has (1X10^{-6}/67000) X46=6.87X10^{-10} moles of DTPA. Therefore, 1 mole of 30XDSR-PL has 6.87X10^{-10}/1.74X10^{-11}=39 moles of DTPA on it.
For **20X DSR-PL**, 1 µg/ml of DTPA-BSA shows equivalent binding to that of 0.008X0.04mg/ml= 0.32 µg/ml of 20X DSR-PL, which equal to 2.32X10^{-11} moles of PL. Since there are 46 lysine residues are modified in the DTPA-BSA (46 moles of DTPA/mole of BSA), 1 µg/ml of DTPA-BSA has (1X10^{-6}/67000)X46=6.87X10^{-10} moles of DTPA. Therefore, **1 mole of 20XDSR-PL** has 6.87X10^{-10}/2.32X10^{-11}=30 moles of DTPA on it.

For the **30X DSR-PL**, the ratio of DTPA: RITC=39:36, which is approximately 1:1. For the **20X DSR-PL**, the ratio of DTPA: RITC=30: 18, which is approximately 2:1.
Figure 19: Schematic of RITC and DTPA on Poly-L-Lysine using MarvinSketch

A) PLL alone  B) 30XDSR-PL  C) 20XDSR-PL  D) Distance between two adjacent RITC on 20X DSR-PL (33.64 angstroms)  E) Distance between two adjacent RITC on 30X DSR-PL (20.44 angstroms)

3.5.6 Zeta potential analysis for the 20XDSR-PL and 30XDSR-PL

<table>
<thead>
<tr>
<th>DSR-PL</th>
<th>Zeta potential at 25 °C (mV)</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X</td>
<td>-18.26</td>
<td>2.30</td>
</tr>
<tr>
<td>30X</td>
<td>-12.53</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Figure 20: Zeta potential analysis for the 20XDSR-PL and 30XDSR-PL after succinylation by succinic anhydride

As seen from Fig. 20, both 20X and 30X DSR-PL are negatively charged after succinylation. Compared to 30XDSR-PL, 20 XDSR-PL is more negatively charged. On 20XDSR-PL, the number of RITC conjugated to poly-lysine is less than that in 30XDSR-PL; therefore, much more free lysyl residues are left on the 20X DSR-PL. Therefore, after succinylation, 20X DSR-PL is more negatively charged than 30X DSR-PL.

3.5.7 HPLC profiles of the 20X, 30X DSR-PL compared to free PL, PGA and D-PGA-Dox

It is shown in Fig. 21-A and B that the 20X DSR-PL was eluted at 8.6 minute by OD 220nm assessment, and 8.9 minutes by the fluorescence detector. The delay between the elution times is due to the tandem connection of the spectrometer and the fluorometer. The elution peak of the 20X DSR-PL by OD 220 nm and fluorescent assessments confirmed that 20X DSR-PL is pure with minimal larger molecular weight contaminating species. It showed not free RITC peak. The 30X DSR-PL HPLC elution profiles also showed the same result (Fig. 21-C and D). Fig. 21-E and F show the difference in the elution profile due to the pH of the elution buffer using polylysine as the sample for size exclusion chromatography. The pH of the elution buffer (0.2M sodium phosphate) was 8.5 in E and the pH of the elution buffer also 0.2M sodium phosphate, was 7.0 in F. The isoelectric point (IP) of poly-lysine is 9.0, therefore, polylysine is less positively charged at the pH of 8.5, resulting in less non-specific ionic interaction with the matrix of the column. The elution time at pH of 8.5 was 11.7 minute compared to 12.5 minute in pH of 7.0, which imparted higher positive charged to poly-lysine (F). Similar effect of charge of the polymers is shown in Fig. 21-G and H. When PGA was conjugated with DTPA and Dox, PGA
became less negatively charged. D-Dox-PGA is eluted later at 10.8 minute compared to the free PGA, which is eluted at 9.2 minute. Therefore, the elution time of positive charged polymers are delayed by HPLC. Whereas succinylation renders the polymer more negatively charged and therefore eluted with an earlier elution time.

3.5.8 Assessment of in vitro fluorescence using purified Canine cardiac myosin:

Canine cardiac myosin or referred to as dog heart myosin (DHM) was previously prepared in our laboratory. An aliquot of 500µl of the solution was added to 5ml of cold distilled H2O to precipitate the myosin. After overnight incubation at 4°C in a refrigerator, DHM precipitate was centrifuged at 3,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the DHM gel precipitate was solubilized in 3 ml of 0.3M PBS. DHM (100µl) aliquots were applied on microscopic slides and smeared on the slides by dragging an edge of another microscope slide along the first slide. Myosin was allowed to dry and then fixed by dipping the slides in -20°C pre-cooled acetone. The slides were dried and blocked with 3% BSA. After washing, the slides were incubated with 1 µg/ml of bsAb+1 µg/ml 20X or 30X DSR-PL, 1 µg/ml anti-myosin-RITC, 1 µg/ml anti-myosin and 1 µg/ml anti-DTPA+ 1 µg/ml GAM-RITC(TRITC). Negative controls consisted of untreated DHM slides, 20X and 30X DSR-PL treated myosin slides without pre-treatment with the bsAb, whereas positive controls consisted of anti-myosin-RITC.(26)

At 1 µg/ml concentration of anti-myosin-RITC, 1 µg/ml anti-myosin+ 1 µg/ml GAM-RITC and 1 µg/ml anti-myosin+1µg/ml GAM-TRITC, no fluorescence was detected indicating that the concentration was too low for epifluorescent visualization at 800 msec exposure and gain of 4. However, pretargeting with bsAb and targeting with 20X DRS-PL and 30X DRS-PL showed epifluorescence. The bsAb+20X DSR-PL treated myosin on the microscopic slides was
significantly more fluorescent than the other control group reagents targeted myosin. No background fluorescence from each epifluormicrograph was subtracted to normalize these micrographs as shown in Fig. 22, however, quantitation of the pixel density of fluorescence shown in Fig. 23 were background fluorescence activity corrected.

Figure 22: Epifluorescence microscopy for in vitro DHM slides stained with different reagents 1) Myosin alone 2) Myosin+1 µg/ml anti-myosin-RITC 3) Myosin+1 µg/ml BsAb+1 µg/ml 20X DSR-PL 4) Myosin+1 µg/ml BsAb+1 µg/ml 30X DSR-PL 5) Myosin+1 µg/ml anti-myosin+1 µg/ml GAM-RITC 6) Myosin+1 µg/ml anti-myosin+1 µg/ml GAM-TRITC 7) Myosin+1 µg/ml anti-DTPA+1 µg/ml GAM-RITC 8) Myosin+1 µg/ml anti-DTPA+1 µg/ml GAM-TRITC 9) only 1 µg/ml 20X DSR-PL 10) only 1 µg/ml 30X DSR-PL

Mean Fluorescent Pixel Density assessment of in vitro fluorescence of the DHM slides after background fluorescence correction
The raw epifluoromicrographs of DHM treated with 1 µg/ml of anti-myosin-RITC, 1 µg/ml of anti-myosin+1 µg/ml of GAM-TRITC, 1 µg/ml of anti-myosin+1 µg/ml of GAM-RITC, 1 µg/ml of anti-DTPA+ 1 µg/ml of GAM-TRITC, 1 µg/ml of anti-DTPA+ 1 µg/ml of GAM-RITC, 1 µg/ml of bsAb+20X DSR-PL, 1 µg/ml of bsAb+30X DSR-PL, only 20X, 30X DSR-PL and only myosin are shown in Fig. 22. No background fluorescence from each epifluoromicrograph was subtracted to normalize these micrographs. Mean fluorescent pixel density was determined by quantitative analysis of the epifluoromicrographs using ImageJ program (National Institutes of Health) however all micrographs were normalized for the background activity (Fig. 23).

3.5.9 Immunofluorescence of frozen murine heart sections (first set):

Ten-micron thick murine frozen heart sections were blocked with 3% BSA for 1 hour at room temperature. Then the slides were washed with 0.1M PBS-T and PBS as described above.

The epifluoromicrographs of frozen murine myocardial sections treated with 1 µg/ml of anti-myosin-RITC, 1 µg/ml of anti-myosin+ 1 µg/ml of GAM-TRITC, 1 µg/ml of anti-DTPA+ 1 µg/ml of GAM-RITC,
µg/ml of GAM-TRITC, 1 µg/ml of bsAb+1 µg/ml of 20X DSR-PL, 1 µg/ml of bsAb+1 µg/ml of 30X DSR-PL and only 1 µg/ml of 20X, 30X DSR-PL are shown in Fig. 24. No background fluorescence correction has been performed in these epifluormicrograph. Mean fluorescent (red) histogram analyses in pixel density determined by quantitative analysis of the epifluormicrographs using ImageJ program from NIH, normalized for background activity is shown in Fig.25.

Each fluormicrograph was assessed for pixel density in 5 separate regions and each treatment was repeated 5 times resulting in n = 25 for each treatment. The mean pixel density for red fluorescence of the section treated with 1 µg/ml of bsAb + 1 µg/ml 20X DSR-PL was 116.6 ± 16.9 which was significantly higher than section treated with anti-myosin-RITC (22.3 ± 10.3) and other control groups. (* indicates p <0.05)

![Figure 24: Epifluorescence pictures for frozen murine cardiac sections stained with different reagents (First set).](image-url)
Figure 25: Mean Pixel Density after background activity correction (+SD) of frozen murine cardiac sections stained with different reagents (First set) * indicates p <0.05.

Pretargeting with BsAb (anti-myosin-anti-DTPA antibody) and specific targeting with fluorescence-loaded 20X DSR-PL demonstrated significantly higher signal amplification and sensitivity in *in vitro* immunofluorescence imaging than in controls.

The same experiment was repeated on two more sets of frozen murine heart sections. The results are as shown in Fig. 26 to Fig. 29. Mean pixel density of the sections treated with 1 μg/ml of bsAb + 1 μg/ml 20X DSR-PL was significantly higher than the sections treated with anti-myosin-RITC and other control groups. (*) indicates p <0.05
Immunofluorescence in frozen murine heart sections (second set):

Figure 26: Epifluorescence pictures for frozen murine cardiac sections stained with different reagents (Second set)

![Immunofluorescence images](image)

Figure 27: Mean Pixel Density after background activity correction (+SD) of frozen murine cardiac sections stained with different reagents (Second set) * indicates p <0.05.
Immunofluorescence in frozen murine heart sections (third set):

Figure 28: Epifluorescence pictures for frozen murine cardiac sections stained with different reagents (Third set)

Figure 29: Mean Pixel Density after background activity correction (+SD) of frozen murine cardiac sections stained with different reagents (Third set) * indicates p <0.05.
Figure 30: Standard curves of bsAb+20X (red line) compared to bsAb+30X (purple line), anti-myosin-RITC (black line) and normal heart (blue line)

Total mean pixel densities from three individual immunofluoresence studies are shown in Fig. 30 (n=75). The bsAb+20X (red line) had significant signal amplification compared to other control groups.
3.5.10 FACS analysis:

Cultured H9C2 cells were cultured in 12 well plates starting with 40,000 cell/well. After 80% confluency, cells were trypsinized. The cells were killed by freezing as described in the method section and treated with different reagents: 1 µg/ml anti-myosin + 10 µg/ml GAM-RITC or GAM-TRITC, 1 µg/ml anti-DTPA + 10 µg/ml GAM-RITC or GAM-TRITC, 1 µg/ml bsAb + 1 µg/ml 20X or 30X DSR-PL, 1 µg/ml 20X or 30X DSR-PL alone, and 5 µg/ml, 1 µg/ml of anti-myosin-RITC. The number of cells and the intensity of cells in each sample were assessed by flow cytometry (Fig. 31).
4) 1 µg/ml anti-DTPA + 10 µg/ml GAM-RITC
5) 1 µg/ml anti-DTPA + 10 µg/ml GAM-TRITC
6) 1 µg/ml bsAb + 1 µg/ml 20X DSR-PL

7) 1 µg/ml bsAb + 1 µg/ml 30X DSR-PL
8) only 1 µg/ml 20X DSR-PL
9) only 1 µg/ml 30X DSR-PL

10) 1 µg/ml anti-myosin-RITC
11) 5 µg/ml anti-myosin-RITC

Figure 31-A: FACS analysis of H9C2 cells treated with different reagents combination: 1) untreated H9C2 cell alone  2) 1 µg/ml anti-myosin+ 10 µg/ml GAM-RITC  3) 1 µg/ml anti-myosin+ 10 µg/ml GAM-TRITC  4) 1 µg/ml anti-DTPA+ 10 µg/ml GAM-RITC  5) 1 µg/ml anti-DTPA+ 10 µg/ml GAM-TRITC  6) 1 µg/ml bsAb+ 1 µg/ml 20X DSR-PL  7) 1 µg/ml bsAb+ 1 µg/ml 30X DSR-PL  8) only 1 µg/ml 20X DSR-PL  9) only 1 µg/ml 30X DSR-PL  10) 1 µg/ml anti-myosin-RITC  11) 5 µg/ml anti-myosin-RITC
Figure 31-B: FACS analysis of H9C2 cells treated with different reagents combination. Positive shift was observed in Data 006) 1 µg/ml bsAb+ 1 µg/ml 20X DSR-PL.

**Histogram of the FACS analysis**

![Histogram of the FACS analysis](image)

Figure 32: Histogram of the FACS analysis: 1) untreated H9C2 cell alone  2) 1 µg/ml anti-myosin+ 10 µg/ml GAM-RITC  3) 1 µg/ml anti-myosin+ 10 µg/ml GAM-TRITC  4) 1 µg/ml anti-DTPA+ 10 µg/ml GAM-RITC  5) 1 µg/ml anti-DTPA+ 10 µg/ml GAM-TRITC  6) 1 µg/ml bsAb+ 1 µg/ml 20X DSR-PL  7) 1 µg/ml bsAb+ 1 µg/ml 30X DSR-PL  8) only 1 µg/ml 20X DSR-PL  9) only 1 µg/ml 30X DSR-PL  10) 1 µg/ml anti-myosin-RITC  11) 5 µg/ml anti-myosin-RITC

From the FACS analyses and histograms (Fig. 31-A, B and 32), 20X DSR-PL has somewhat higher non-specific background fluorescence (6.39), however, it is still significantly
lower than that of 20X DSR-PL pretargeted with bsAb (15.8). Even after background subtraction, the signal of pretargeting with bsAb followed by targeting with 20X DSR-PL is significantly greater than the rest and has the highest signal compared to controls. Signal of cells treated with bsAb and targeting with 1 µg/ml 20X DSR-PL was still five folds higher than the signal of cells treated with 5µg/ml anti-myosin-RITC.
4. Discussion:

After the bsAb (anti-myosin-anti-DTPA) appears to be stable. Assessment of the immunoreactivities of previously made bsAb showed that there was no loss of immunoreactivity of the individual antibodies. Structural stability was demonstrated by SDS-PAGE in Fig.10. After RITC was conjugated to GAM and anti-myosin antibody, we also demonstrated that there was no loss of the immunoreactivity as GAM-RITC or anti-myosin-RITC.

Size exclusion chromatography of 20X and 30X DSR-PL using Sephadex G-25 column (1x10 cm) also showed that the polymer preparations did not contain contaminating free fluorophores. However, this size exclusion chromatography cannot ascertain whether the polymers eluted in the void volume contained all fluorophore-modified polymers or whether unmodified polymers are also present. There is no free RITC in the 20X and 30X DSR-PL preparations.

Table I shows the TNBS result representing modification of lysyl residues on BSA. Based on the modification rate of BSA and ELISA results in Fig.18, the concentration of DTPA on 20X, 30X DSR-PL was determined to be 30 and 39 moles DTPA per mole of 20X and 30X DSR-PL respectively. The moles concentration of Rhodamine B in 20X and 30X DSR-PL was 18 and 36 respectively. Therefore, it appears that there are approximately 2 moles DTPA per mole of Rhodamine B on the 20X DSR-PL, and approximately equimolar concentration of DTPA and rhodamine B in 30X DSR-PL. Using these values of moles equivalence, a model of the DSR-PL polymers was generated in Fig.19 D and E. In a helical conformation with half a complete turn of 180 degrees for every 4 lysyl residues, we propose that the two adjacent Rhodamine B in 20X DSR-PL are oriented 180 degrees from each other on opposite sides with 2 DTPA molecules between them. Whereas in the 30X DSR-PL, two
Rhodamine B molecules are spaced by only 1 molecule of DTPA resulting on only a 135 degrees turn of the helix. The distance between the adjacent rhodamine molecules on 30 X DSR-PL is approximately 20.44 angstroms, separated by only one DTPA. The distance between the adjacent rhodamine molecules on 20 X DSR-PL is approximately 33.64 angstroms, separated by two DTPA. The closer proximity probably resulted in higher self-quenching even though the fluorescent intensity of 30X DSR-PL pretargeted with bsAb was higher than the conventional rhodamine B labeled antibodies. When the two adjacent fluorphores are opposite in the direction of the turn of the helix of the polylysine, the distance is much greater which may explain the enhanced fluorescent intensity of greater than hundreds folds relative to the standard fluorophore labeled antibodies. Furthermore, polylysine used is a Poly-L-lysine hydrobromide. The 13,800Da polylysine HBr consists of about a mean of 66 lysine residues (Sigma Aldrich package insert). Therefore there should be a mean of 66 amino groups and 1 N-terminal amino residue. In our 20X DSR-PL, approximately 48 amino groups are modified with either DTPA or rhodamine B. In the 30X DSR-PL, almost all amino residues are modified with equimolar concentration of DTPA (39 DTPAs) and rhodamine B (36 rhodamines). It is not possible to conjugate 75 moles of DTPA and rhodamine B per mole of polylysine. This estimate of the modification of 30X DSR-PL is an overestimation. However, the molecular weight is also an estimate of the mean of a Gaussian distribution of the polymers. Assuming that the DTPA and rhodamine-B concentrations in the 20X DSR-PL are reasonable, the model provided showing the placement of the rhodamine-B fluorophores and DTPAs becomes plausible. The separation of the rhodamine-B fluorophores by 2 DTPAs therefore allows sufficient distance to overcome the phenomenon of quenching observed when two or more fluorophores are in close proximity on the detection reagents.
The Zeta potential of both 20X and 30X DSR-PL are negative after complete succinylation. 20 XDSR-PL is more negatively charged than that of 30X DSR-PL. However, it appears from the immunofluormicrographic images that 20X DSR-PL has higher non-specific background activity that that with 30X-DSR-PL. The rationale based only on charge to reduce non-target background activity may not be sufficient. Even thought the Zeta potential of 20X-DSR-PL is -18.26±2.3 mV and that of 30X DSR-PL is -12.52±2.0 mV, at this time, we do not have a plausible explanation for this observation. One possibility is that concentration of bovine serum albumin used to block the slides is 3%, which is not high enough for blocking, and the glass slide maybe positively charged to facilitate the adhesion of tissue section. This may allow more negatively charged 20X-DSR-PL to bind with higher non-specific background activity. The less negatively charged 30X DSR-PL showed less non-specific background activity. If this explanation was correct, blocking with higher concentration of BSA should show decreased non-specific background activity. Such an experiment shall be the investigation of the next master’s student project.

FACS analyses confirm our observation that pretargeting with bsAb and targeting with 20X and 30X DSR-PL resulted in greater fluorescent signal than with standard immunofluorophore labeled antibodies. Therefore, my master’s thesis research shows that pretargeting with bsAb and targeting with fluorophore conjugated polymers that are designed to space the adjacent fluorophores on opposite directions of the helical turn of the polylysine resulted in prevention of fluorescence quenching and amplification of the fluorescent signal.

This approach of signal amplification may be applicable to detection of other biomarkers such as tumor, neurological or cardiovascular biomarkers.
5. Conclusion:

The usage of directly labeled monoclonal antibodies with fluorophores is limited by the nonspecific accumulation leading to a low target to background ratios. RITC is widely used as a fluorescent tag in biological experiments. However, its major shortcoming is its self-quenching effect when high specific activity reagents are needed.

Pretargeting with anti-myosin-anti-DTPA bispecific antibody followed by 20X DSR-PL for immunofluorescent imaging of canine heart myosin coated microscope slides, frozen murine myocardial sections, and for FACS analyze of permeabilized H9C2 rat embryonic cardiomyocyte demonstrated significantly higher signal amplification and sensitivity due to reduction of quenching of fluorescence with RITC fluorophores when multiple Rhodamine fluorophores are conjugated on the molecule. Decreasing the phenomenon of self-quenching and signal amplification can be achieved at the same time by targeting with 20X DSR-PL after pretargeting with bispecific antibody. Furthermore, by using various molar concentration of DTPA and RITC, better signal polymer may be generated where the 2 moles of RITC may be conjugated with 6 moles of DTPA that would produce adjacent RITC for every turn of the polylysine helix spaced by 6 DTPA.
6. References:


   http://www.drmr.com/abcon/FITC.html
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