Video Article

The Use of the *Ex Vivo* Chandler Loop Apparatus to Assess the Biocompatibility of Modified Polymeric Blood Conduits

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Abstract

The foreign body reaction occurs when a synthetic surface is introduced to the body. It is characterized by adsorption of blood proteins and the subsequent attachment and activation of platelets, monocyte/macrophage adhesion, and inflammatory cell signaling events, leading to post-procedural complications. The Chandler Loop Apparatus is an experimental system that allows researchers to study the molecular and cellular interactions that occur when large volumes of blood are perfused over polymeric conduits. To that end, this apparatus has been used as an *ex vivo* model allowing the assessment of the anti-inflammatory properties of various polymer surface modifications. Our laboratory has shown that blood conduits, covalently modified via photoactivation chemistry with recombinant CD47, can confer biocompatibility to polymeric surfaces. Appending CD47 to polymeric surfaces could be an effective means to promote the efficacy of polymeric blood conduits. Herein is the methodology detailing the photoactivation chemistry used to append recombinant CD47 to clinically relevant polymeric blood conduits and the use of the Chandler Loop as an *ex vivo* experimental model to examine blood interactions with the CD47 modified and control conduits.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51871/>

Introduction

Many clinical procedures, such as cardiopulmonary bypass and renal dialysis, require the use of polymeric blood conduits and are often associated with post-procedural complications¹. When perfused with blood, these polymers illicit the foreign body reaction (FBR), resulting in adsorption of blood proteins and platelets, monocyte/macrophage adhesion, and the release of pro-inflammatory cytokines, all of which contribute to post-procedural complications and/or device failure^{2,3}. Thus, strategies to address this issue remain an important and ongoing area of biomaterials research. Investigators have attempted to address this issue by modifying blood contacting surfaces with bioactive or bioinert molecules⁴⁻⁶. Research in our laboratory has focused on appending recombinant CD47 (recCD47) to polymeric biomaterials as a strategy to mitigate the FBR and increase the efficacy of these materials. CD47 is a ubiquitously expressed transmembrane protein with a known role in immune evasion, conferring "self" status upon expressing cells⁷⁻¹⁰ and shows promise at conferring biocompatibility when appended to polymeric surfaces¹¹⁻¹³. Signal-regulatory protein alpha (SIRPα), the cognate receptor for CD47, and a member of the immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing family of transmembrane proteins, is expressed on cells of myeloid origin¹⁴. We have previously demonstrated that CD47, via SIRPα-mediated cell signaling, down-regulates the immune responses to polyurethane (PU) and polyvinylchloride (PVC) in *in vitro*, *ex vivo*, and *in vivo* models¹¹⁻¹³.

Central to our investigations is a relatively novel photoactivation chemistry, described herein, in which chemically reactive thiol groups are covalently appended to polymeric tubing by reacting the tubing with a multifunctional polymer (PDT-BzPh), composed of 2-pyridyldithio (PDT), the photoreactive benzophenone (BzPh) and a carboxy-modified polyallylamine¹¹⁻¹³. Reducing the covalently appended PDT groups with tris(2-carboxyethyl) phosphine hydrochloride (TCEP)¹¹ yields a thiolated surface that can be subsequently reacted with therapeutic moieties. Detailed herein and previously^{12,13}, recCD47, further modified with the addition of a C-terminal poly-lysine tail^{12,13}, is reacted with Sulfo-succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) for 1 hr to generate thiol-reactive groups, allowing for a monosulfide bond formation between the tubing and recCD47¹¹. The anti-inflammatory capacity of the CD47 functionalized surfaces was tested, *ex vivo*, using the Chandler Loop Apparatus with whole human blood, which was originally described in 1958 as an *in vitro* model of thrombotic coagulation¹⁵. The apparatus relies on a closed tube system partially filled with air and a rotary motor to circulate the blood through the tubing¹⁵. This experimental model provides the opportunity to examine the effect of blood exposure upon modified and unmodified surfaces as well as the effect of those surface modifications upon the physiology of cells of the blood.

recCD47 can be appended to a variety of polymeric surfaces using this photoactivation chemistry, and its anti-inflammatory capacity can be assessed by utilizing a clinically relevant *ex vivo* model mimicking blood perfusion over polymeric surfaces^{11,12}. Clinical grade blood conduits

modified with recCD47 show significantly less platelet and inflammatory cell attachment as compared to unmodified polymers when exposed to human blood in the apparatus. A step-by-step description of this modification process is detailed below.

Protocol

1. Modifying Polymeric Surfaces with recCD47

NOTE: The protocol is summarized schematically in **Figure 1**. **Figure 1A** illustrates generation of thiol-reactive polymeric surfaces. **Figure 1B** illustrates generation of thiol-reactive recCD47.

1. Day 1
 1. Prepare a solution of PDT-BzPh (1 mg/ml) and potassium bicarbonate (KHCO_3) (0.7 mg/ml) in sterile water. Stir overnight at 4 °C (*protect from light*).
2. Day 2
 1. Cut polymeric tubing into 40 cm long pieces (long enough to fit around rotating wheels).
 2. Soak tubes in a 0.1% aqueous solution of hexacylpyridinium for 90 min at room temperature on a shaker or on Chandler Loop Apparatus. Rinse the tubes with sterile water 3x after the 90 min soak.
 3. Acidify the solution of PDT-BzPh from Day 1 by adding 15% aqueous potassium phosphate monobasic (KH_2PO_4). Add 50 μl KH_2PO_4 per ml of PDT-BzPh, forming a cloudy micellar solution.
 4. Soak the tubes in the acidified PDT-BzPh solution for 40 min at room temperature on a shaker or on apparatus.
 5. Rinse the tubes with dilute acetic acid (1:1,000) once.
 6. Expose the tubes to UV irradiation for a total duration of 60 min. Rotate tubes $\frac{1}{4}$ turn every 15 min to irradiate the entire surface area.
 7. Soak the tubes in a solution of 20 mg/ml potassium bicarbonate (KHCO_3) for 20 min at room temperature on a shaker or on apparatus.
 8. Rinse the tubes with sterile water 3x and store at 4 °C in sterile water.
3. Day 3
 1. Dissolve 5 mg Sulfo-SMCC in 200 μl Dimethylformamide (DMF) (*Caution: Perform in fume hood*).
 2. Add 50 μl of the Sulfo-SMCC solution prepared in step 1 to 0.1 mg/ml of recCD47 poly-lysine solution, and stir for 60 min at room temperature.
 3. During 60 min stir, degas sterile Dulbecco's Phosphate Buffered Saline (DPBS) and set aside.
 4. Purify Sulfo-SMCC reacted recCD47 using a 7K molecular weight cut-off spin desalting column per manufacturer's instructions. Collect final flow-through (high-quality thiol-reactive recCD47) and dilute to a volume necessary to coat the interior of the tube with DPBS.
 5. Rinse the tubes from day 2 with sterile, degassed DPBS 3x.
 6. React the modified surface of the tubes with a solution of 20 mg/ml with tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in degassed DPBS for less than 2 min. Rinse the tubes with sterile degassed DPBS 4x.
 7. Add the Sulfo-SMCC-reacted recCD47 to the tubes and incubate overnight at 4 °C on a shaker or on apparatus.

2. Immunoassay Quantification of recCD47 on Modified Surfaces

1. Rinse modified tubes to be quantified from Day 3 with DPBS 3x. Store tubes not used for quantification in DPBS at 4 °C.
2. Use a 4 mm biopsy punch to make duplicate tube samples and place biopsy punches in wells of a 96-well plate.
3. Prepare a negative control consisting of an unmodified tube sample not treated with the detection antibody. Prepare an antibody control consisting of an unmodified tube sample treated with the detection antibody. Prepare the modified tubing test samples treated with the detection antibody.
4. Block samples with 0.4% bovine serum albumin (BSA) in DPBS for 60 min at room temperature on a shaker.
5. After blocking, aspirate BSA and rinse with tris-buffered saline plus 1% Tween-20 (TBST) 3x, 10 min each on a shaker.
6. Prepare a working dilution of antibody in 0.4% BSA according to manufacturer's recommended dilution for immunoassays. Dilute human CD47 Antibody B6H12-FITC 1:100 in 0.4% BSA.
7. Add 200 μl of antibody dilution to appropriate wells. Incubate negative controls with 0.4% BSA only. Incubate at room temperature for 60 min on a shaker (*protect from light*).
8. Rinse with TBST 3x, 10 min each on a shaker. Aspirate the last TBST rinse and add 200 μl DPBS to tube sample wells.
9. Collect the final flow-through, which is high-quality thiol-reactive recombinant CD47 and dilute to a volume necessary to coat the interior of the tubes with DPBS.
10. Read FITC signal intensity using a microplate reader with FITC excitation (485 nm) and emission (538 nm) settings.
11. Calculate recCD47 bound to polymeric surface based on standard values, accounting for auto-fluorescence and non-specific binding from the antibody control sample.

3. Chandler Loop Apparatus Protocol

Seek Institutional Review Board (IRB) approval of blood collection protocol and informed consent paperwork prior to initiation of collection of human blood samples. Obtain informed consent from a human blood donor.

NOTE: A diagram depicting the apparatus is shown in **Figure 2**.

1. Fill water bath with distilled water until approximately 1/2 of the diameter wheels are submerged. Add enough bleach to the water bath to make a 10% bleach solution. Set water bath to 37 °C and allow temperature to equilibrate.

2. Gather metal adapters (shown in **Figure 1B**), unmodified tubing and modified tubing, and assemble around apparatus wheels, as shown in **Figure 1C**. Ensure that tubing fits snugly around the wheel with the metal adapter in place.
3. Obtain 30 ml blood sample using an IRB-approved protocol, in a vial prefilled with 2 ml of citrate or other anticoagulant, and mix by inversion to prevent clotting once the specimen has been collected.
4. Add approximately 10 ml of blood to each tube using the metal valve and syringe, leaving some air in the tube. Secure the valve cap and rotate for 3 hr.
5. Drain the blood into a waste beaker treated with a 10% bleach solution (or as required by institutional policy).
6. Gently rinse tubing interior with DPBS to remove all traces of blood. Collect flow-through in the waste beaker. Dispose of blood in accordance with institutional policy.
7. Disinfect the apparatus and any blood contacting surfaces using a 10% bleach solution or according to institutional policy.
8. Process samples for fluorescent microscopy or scanning electron microscopy as described below.

4. Fluorescent Microscopy and Cell Counting

1. Prepare a 4% paraformaldehyde (PFA) solution (*Caution: Perform in fume hood*).
2. Incubate tubing in a 4% PFA solution overnight at 4 °C.
3. After overnight incubation, remove 4% PFA and rinse films very gently with DPBS.
4. Cut the tubing into sections to expose the lumen surface for staining.
5. Stain a section of the tubing with a few drops of mounting media with 4',6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature (*protect from light*). After staining, rinse the tubing very gently with DPBS to reduce background DAPI signal.
6. Image using a fluorescent microscope equipped with a DAPI filter and digital camera to count the number of cells in 9 blindly selected fields of view under 200X magnification.
7. Record cell counts per field of view and perform appropriate statistical analyses to determine statistical significance of results.

5. Scanning Electron Microscopy

1. Prepare a 2% glutaraldehyde solution (*Caution: Perform in fume hood*).
2. Incubate tubing in a 2% glutaraldehyde solution overnight at 4 °C.
3. After overnight incubation, gently rinse the tubing 3x with DPBS.
4. Prepare a 1% solution of osmium tetroxide (*Caution: Severe inhalation hazard! Use in fume hood*).
5. Incubate tubing in a 1% solution of osmium tetroxide for 15 min at room temperature.
6. Gently rinse the tubing 3x with DPBS.
7. Prepare a series of ethanol concentrations (25%, 50%, 75%, 95%, and 100% ethanol).
8. Dehydrate the tubing by incubation in the series of ethanol concentrations. Incubate in 25%, 50%, 75%, and 95% ethanol for 20 min each. Incubate in 100% ethanol for 30 min.
9. Supercritical point dry the tubing with CO₂ for 45 min to remove all moisture.
10. Mount tube sections on a specimen stub with silver paste or graphite.
11. Coat tube sections with 12.5 nm of gold-palladium.
12. Examine in a scanning electron microscope.

Representative Results

Generating thiol-reactive polymeric surfaces through the use of PDT-BzPh and TCEP along with thiol-reactive recCD47 poly-lysine using SMCC allows for the attachment of recCD47 to polymeric surfaces. The modification process is summarized schematically in **Figure 1**. The convenience of this modification process is that it can be applied to many different proteins and many different polymeric surfaces, assuming the protein can be modified with sufficient chemically reactive groups such as amine-containing lysines and that the polymer has sufficiently available hydrocarbons.

It has been demonstrated previously that recCD47 can be appended to polymeric surfaces using this protocol¹¹⁻¹³. As shown in **Figure 3A**, significant FITC staining is visible for recCD47, using a FITC-conjugated antibody to the extracellular Ig domain of CD47, which is localized specifically to the film as shown by DIC imaging (**Figure 3B**). These results demonstrate that recCD47 can be covalently attached to polyurethane films. Measuring the fluorescent units and comparing with a standard curve determined the concentration of surface bound recCD47. We have shown that recCD47 can be appended to polymeric surfaces at levels exceeding 500 ng/cm^{2,11-13}. These results confirm that this thiol-based polymer modification protocol can be used to append poly-lysine modified recombinant proteins to polymeric surfaces.

CD47 has been shown to be a marker of "self," preventing inflammatory cell attachment and immune system activation⁷⁻¹³. To mimic *in vivo* conditions as closely as possible in an *ex vivo* setting, whole human blood can be perfused through unmodified and modified polymeric tubing in the Chandler Loop Apparatus (shown in **Figure 2**). Cell attachment to the tubing can be assessed via DAPI staining (**Figure 4**) and SEM (**Figure 5**). Cell counts obtained through DAPI staining demonstrate that appended recCD47 significantly ($p = 0.004$) inhibits cell attachment compared to unmodified surfaces (**Figures 4A & 4B**). The DAPI cell counts were confirmed by SEM, demonstrating similar levels of cell attachment to unmodified and recCD47 modified surfaces (**Figure 5**). These data indicate that appendage of recCD47, using the protocol described herein, significantly inhibits inflammatory cell attachment in an *ex vivo* model of blood perfusion.

Figure 1. Schematic of Surface modification. A) Thiolated polymer surfaces were generated by incubating the polymeric surface with the photoactivatable crosslinker PDT-BzPh and subsequent reduction with TCEP. B) SMCC was used to produce thiol-reactive recCD47, which was then reacted with the thiolated synthetic surface to yield recCD47 functionalized surfaces.

Figure 2. Diagram of Chandler Loop Apparatus. A) The apparatus consists of a water bath heated to 37 °C, rotating wheels fixed on a metal pole attached to a rotary motor. This set up allows for the rotary motor to turn the wheels, thereby submersing portions of the tubing into the 37 °C water bath and perfusion of blood through the tubing. B) Metal adapters are used to connect the ends of the tubing forming a loop around one of the rotating wheels. Blood is added to the tubes through the metal valve port and capped with the valve cap. C) Once assembled, the tubing and metal valve should fit snugly around the rotating wheel, as shown here with an empty tube.

Figure 3. Quantization of recCD47 on polyurethane films. Antibodies directed against the external Ig domain of CD47 were used to quantify the amount of recCD47 bound to polyurethane films. Fluorescent microscope images taken under 200X magnification with appropriate filter sets showing FITC detection of recCD47 appended to polyurethane (**A**). (**B**) DIC images show that FITC signal is specific to the polyurethane film.

Figure 4. Cell adhesion to unmodified and recCD47-modified polyurethane. Whole human blood was collected and perfused over unmodified or recCD47-modified PU films for 3 hr. Following the 3 hr perfusion, films were rinsed with DPBS, and the adhered cells were fixed with 4% PFA overnight at 4 °C and DAPI stained for 30 min at room temperature. DAPI-stained cells were counted using a fluorescent microscope under 200X magnification and appropriate filter sets. **(A)** 9 randomly selected fields of view were counted for each modification. **(B)** Data is representative of 9 fields of view and is expressed as mean ± standard error ($p = 0.004$).

Figure 5. SEM analysis of recCD47 modified and control surfaces following ex vivo analysis. Whole human blood was collected and perfused over unmodified or recCD47-modified PVC tubing for 3 hr. Representative images show significant cell attachment to unmodified surfaces while recCD47-modified surfaces show only an occasional attached cell.

Discussion

The photoactivation chemistry (summarized in **Figure 1**) allows for the modification of virtually any polymer surface that has sufficient hydrocarbons to facilitate PDT-BzPh attachment and subsequent UV irradiation to photo-activate the PDT-BzPh. Functionalizing the polymeric

surface with reactive thiol groups allows for the subsequent attachment of a range of testable molecules of interest. In our particular studies we chose recombinant CD47¹¹⁻¹³. The particular conjugation chemistry that we used involved the reaction of the protein's amine groups with the bifunctional cross linker SMCC¹¹⁻¹³. This provided thiol-reactive recCD47 for the subsequent reaction with the thiolated polymeric surface. The end product was a mono-sulfide linkage covalently binding the recCD47 to the polymer. To help ensure that the amine groups from the amino acids in CD47 were not being used for the reaction, and thereby mitigating the efficacy of the immobilized CD47, we further modified the recCD47 by inserting a poly-lysine tag at the protein's C-terminus. It would be feasible that a similar molecular strategy can be applied with other proteins. Thus the conjugation chemistry presented here and elsewhere¹¹⁻¹³ provides an important opportunity to assess the ability of potentially therapeutic molecules to confer biocompatibility upon synthetic surfaces.

A disadvantage to this protocol is that exposing certain types of polymers, such as polyurethane, to extended periods of UV irradiation can result in discoloration. This can be avoided by placing the polymer in polystyrene dishes (commonly used in tissue culture) to prevent discoloration of the polymer without hindering photoactivation of the PDT-BzPh. The wavelength of UV light used in this protocol is 302 nm; deviation from this wavelength can result in inefficient photo-activation of PDT-BzPh. If using a different wavelength of UV light, optimization of UV exposure time is necessary to ensure significant photo-activation.

Following this protocol allows for the attachment of recCD47 to polymeric surfaces, for the purpose of immune evasion and conveying "self" status to the polymeric surface to prevent the foreign body reaction. Quantification of the recCD47 appended to the surface is completed using a FITC-labeled antibody to the Ig domain of CD47 using an immunoassay and fluorescent microscopy (**Figure 3**). This methodology could be adapted to other proteins appended to polymeric surfaces assuming antibody availability. Failure to obtain significant appendage of recCD47 (or other protein) could be due to a variety of factors including, failure to photoactivate PDT-BzPh, insufficient free hydrocarbons on the polymer surface, hydrophobicity of polymer surface, or the polymer could be too thick or opaque to allow light to pass through for adequate quantification. All of these variables can be experimentally tested and optimized for a particular polymer.

The Chandler Loop Apparatus is a unique tool for the *ex vivo* analysis of whole human blood exposure to polymeric surfaces. This system closely resembles the perfusion of blood through clinical blood conduits used in various medical procedures, enabling the analysis of many physiological endpoints associated with blood contacting surfaces. As physiological endpoints for the appendage of recCD47 to polymeric surfaces, cell counts using DAPI staining (**Figure 4**) and scanning electron microscopy (**Figure 5**) were used. Other endpoints can also be used, depending on equipment availability, for example, cell counts before and after blood perfusion through polymeric blood conduits could also be used. Regardless, recCD47-modified surfaces exhibited significantly less adhered cells compared to unmodified control surfaces. These data suggest that recCD47 conveys biocompatibility to polymeric surfaces and could potentially be used to prevent the FBR in clinical procedures using polymeric blood conduits.

Although this is a widely used *in vitro* model for blood perfusion studies, it is limited in some respects. The two major disadvantages of this method arise from the requirement to include air in the tube with the blood sample. The frequent blood interaction with air can cause leukocyte and platelet aggregation and protein denaturation¹⁶⁻¹⁸, which may interfere with certain endpoints. Secondly, the air remains at the highest point of the circuit, limiting the rate of blood circulation¹⁹. Despite the apparent limitations of this method, it induces less blood damage than competing methods¹⁹ and serves as a way of screening the biocompatibility of potential biomolecules on polymeric blood conduits. Once candidate biomolecules are identified through this method, further analysis can be obtained through *in vivo* animal models. Ultimately, the biocompatibility of modified polymers needs to be confirmed using a suitable *in vivo* model system.

Disclosures

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