11-22-2016 12:00 AM

Characterization of N-butyl Cyanoacrylate (NBCA) Glue Polymerization for the Embolization of Brain Arteriovenous Malformations (AVMs)

Bill H. Wang, *The University of Western Ontario*

Supervisor: Dr. Stephen P. Lownie, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Medical Biophysics

Abstract:

Brain AVMs are abnormal connections between arteries and veins. Endovascular glue embolization with NBCA is an accepted form of treatment. The reported complication rates of NBCA embolization varies widely from 2-15%, and timing of polymerization plays a major role.

Polymerization time was measured for mixtures of lipiodol/NBCA of 50/50, 70/30, 60/40. The influence of pH, temperature and presence of biological catalysts on polymerization time was investigated in-vivo. PVA-C, silicone and endothelium surfaces were compared. High-speed video analysis of glue injection through a microcatheter was performed.

Polymerization rate increases with pH and temperature. A hydrophilic surface such as PVA-C better mimics endothelium. Biological substrates including endothelium and blood products dramatically increase the polymerization rate. Characterization of coaxial flow shows dripping to jetting transition with significant wall effect.

The determinants of NBCA polymerization rate are multifactorial and dependent mainly upon the presence of biological substrates coupled with flow-related wall interaction.

Word count: 148

Keywords:

Glue, polymerization, arteriovenous malformations, n-butyl cranoacrylate, NBCA, embolization

Acknowledgements

I would like to thank the following individuals for their support with this project

Thesis Supervisor:

Stephen P. Lownie, MD[1,2,3]

Thesis Advisory Committee:

Melfort Boulton, MD, PhD[1,2,3]

David Pelz, MD[2]

Gord Campbell, PhD[3]

Tamie Poepping, PhD[3]

Lab Coordinator:

Lynn Denning[1]

1. *Department of Clinical Neurological Sciences, London Health Sciences Centre*
2. *Department of Medical Imaging, London Health Sciences Centre*
3. *Department Medical Biophysics, Western University*

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List of Abbreviations

ANOVA – Analysis of variance

ASTM – American Society for Testing and Materials

AVM – Arteriovenous malformation

D5W – 5% dextrose in water (solution)

EVOH – Ethylene vinyl alcohol copolymer

FDA – Food and Drug Administration

NBCA – n-butyl cyanoacrylate

NS – Normal saline

PVA-C – Polyvinyl alcohol cryogel

RBC – Reb blood cell

RHV – Rotatable hemostatic valve

RL – Ringer's lactate

SRS – stereotactic radiosurgery

TCD – Transcranial Doppler ultrasound

General Introduction/Background

Natural history of AVMs:

Brain arteriovenous malformations (AVMs) are abnormal connections between arteries and veins without an intervening capillary bed.[1, 2] The prevalence of brain AVMs in the general population is not precisely known. Previous studies reported numbers that range from 2 to 20 per 100,000 people.[1-3] Some AVMs are asymptomatic while others are symptomatic and cause brain hemorrhage (50%), seizures (40%), neurologic deficits (12%) and chronic headache (14%).[4] Asymptomatic AVMs are discovered either incidentally during neurological imaging for unrelated reasons or at the time of autopsy. Asymptomatic AVMs are more common with an estimated prevalence of approximately 10-20 per 100,000 people.[1, 3] Symptomatic AVMs, on the other hand, occur with a lower frequency of approximately 1-3 per 100,000 people.[2, 3]

Approximately half of all patients with symptomatic AVMs present with an intracerebral hemorrhage.[4-6] In the pediatric population this number is even higher, approaching 80%.[7] Based on natural history studies, the annual risk of hemorrhage from an AVM is around 2-4% per year.[6, 8-11] The biggest risk factor associated with AVM hemorrhage appears to be a history of prior hemorrhage. AVMs that have ruptured have a higher re-rupture rate in the first year of 6-13%, and the hemorrhage rate returns to baseline thereafter at 4% per year or lower.[4, 5, 8, 9, 11] Other factors that increase the risk of hemorrhage include deep venous drainage, existence of associated aneurysms, venous stenosis and deep/infratentorial location.[6, 8-11] AVM nidus size is an inconsistent risk factor, with some studies showing smaller AVMs associated with higher risk of hemorrhage while other studies showing larger AVMs to be more risky.[9, 11] Some studies looking at AVM feeding artery pressure suggest that smaller AVMs are associated with higher feeder pressure, offering a potential explanation for their higher risk of rupture.[12, 13] Larger AVMs, on the other hand, tend to have higher flow rates and potentially expose the fragile “arterialized” veins to higher

hemodynamic stresses, thereby providing an alternate explanation for their propensity to rupture.[11, 13, 14]

Brain AVM rupture is a rare cause of intracerebral hemorrhage, accounting for 1.4-2% of all hemorrhagic strokes;[1, 10, 15] however, in the young adult population (age 18 to 45), it accounts for 25-35% of all hemorrhagic strokes.[6, 16] Hemorrhage is arguably the most serious problem caused by AVMs, and it can have potentially life-threatening consequences for some patients. Mortality after hemorrhage from a brain AVM is reported to be around 10% while residual major disability occurs in 20%–30% of patients.[6, 8-11]

Treatment of AVMs:

Brain AVMs can be treated with open surgery, endovascular embolization, or radiation. All these treatment modalities share a common goal, which is to obliterate the abnormal connections between the feeding arteries and draining veins.[17, 18] Surgery provides the most direct approach, while endovascular therapy attempts to disrupt blood flow to the nidus from within the feeding vessels. Radiation, in the form of stereotactic radiosurgery (SRS), induces endothelial proliferation and results in progressive nidus occlusion over time. Unlike surgery or embolization which exerts its effect immediately, cure from SRS typically occurs slowly, exhibiting a plateau at 3-5 years out (up to 8 years in select patients). The AVM cure rate from SRS varies widely depending on a number variables. In carefully selected patients, it could be as high as 70-80%.[19, 20] Interestingly, endovascular embolization prior to SRS decreases the rate of occlusion at 3 years to approximately 40%.[19]

Symptomatic AVMs (hemorrhage or progressive neurologic deficit) are typically treated with surgery and/or endovascular therapy. Endovascular embolization using liquid embolic agents is an accepted form of treatment of brain AVMs.[18, 21] It is most often used as an adjunct to surgery to reduce perioperative blood loss, although it is sufficient as a stand-alone treatment in approximately 5-10%

of cases.[18, 22] Previous authors have identified certain AVM characteristics associated with higher likelihood of embolization cure such as: small AVM nidus size, less than 3 feeders and superficial feeder/AVM location.[22] When these characteristics are used to screen patients, the cure rate of brain AVMs with embolization alone is higher, ranging from 20% to over 50%.[21-26] This variability is likely related to selection bias and to the lack of rigorous definition of embolization goals – i.e. curative intent versus preoperative devascularization.[22]

Two liquid embolic agents have found widespread use in the clinical setting: N-butyl cyanoacrylate (NBCA) and Ethylene vinyl alcohol copolymer (EVOH, known commercially as Onyx®). These are the only FDA approved liquid embolic agents for neuro-interventional use. The reported complication rate with NBCA embolization varies between 2% to 15%.[21, 23, 25] Hartmann et al. reported in their series of 233 patients treated with NBCA embolization an overall complication rate of 14% and major disability/mortality rate of 3%.[21] Some authors have suggested that most of the complications that occur during embolization with NBCA are related to polymerization rate. [18, 21] A rate that is too fast may cause proximal feeder artery occlusion. Glue could then reflux back into normal upstream vessels, causing stroke. Conversely, a rate that is too slow could cause the glue to occlude the draining vein, or embolize to the lung.[27] Premature venous drainage occlusion may raise the AVM intranidal pressure and trigger intracranial hemorrhage.[24, 28, 29]

The scientific literature reports a wide range of polymerization rates with large discrepancies between in-vivo and in-vitro results.[18, 30, 31] Furthermore, the interaction between glue and vessel surface, as well as the presence of biologic catalysts are poorly understood. Despite its clinical use for over 4 decades (FDA approved in 2000 with clinical use dating almost 3 decades prior), there is still incomplete understanding of NBCA polymerization and injection behavior.[18] This has resulted in large variability amongst authors with respect to glue concentration (typically modified by altering glue/Lipiodol or glue/acetic-acid ratio) and the injection technique (sandwich, wedge or full-column technique) used for NBCA embolization procedures.[18, 21]

Onyx (Covidien, USA) is becoming increasingly popular (FDA approved in 2003) amongst many neuro-interventionalists due to its perceived margin of safety during injection. Onyx has a higher viscosity and slower polymerization rate (more accurately, polymer condensation rate). Therefore, injection can be made over a longer period of time than NBCA with time scales measured in minutes rather than seconds.[18] However, the actual treatment morbidity and mortality is not significantly lower compared to NBCA. Despite the lack of good quality randomized controlled trials, numerous case series have reported overall complication rates between 1% and 17%.[22, 26, 32, 33] Katsaridis et al. reported in their series of 101 AVMs treated with Onyx embolization an overall complication rate of 11% and mortality rate of 3%.[26]

Onyx is unsuitable for high-flow lesions (e.g. AVMs with large fistulous components) because polymerization must occur over a relatively short period of time. In addition, embolization with Onyx in lenticulostriate or thalamoperforating arteries is associated with a high rate of complication (as much as 50%[24]) and can only be accomplished with NBCA. The amount of NBCA operator experience has gradually decreased since the introduction of Onyx. In situations where NBCA is the only option for AVM embolization, the treatment risk for patients is further exacerbated given the incomplete understanding of NBCA polymerization behavior.

Liquid embolic agent chemistry:

NBCA belongs to the family of cyanoacrylate glues which were first synthesized in 1949 and first reported as a tissue adhesive about 10 years later.[34] Figure i. illustrates the chemical formula of various types of cyanoacrylate monomers with different side chain groups (methyl, ethyl, butyl and octyl).[35] The length of side chain determines the polymerization rate, with longer side chains associated with a slower reaction rate. The final polymer formed also becomes more flexible and more resistant to biological degradation as the side chain length increases. For biological use, butyl (NBCA, Histoacryl® – Braun, Germany) or octyl (Dermabond® - Ethicon, USA) sidechains offers better

mechanical matching of tissues and avoids the brittle polymer chains formed by methyl or ethyl (Krazy Glue®) side chains. Cyanoacrylates decompose in the body via hydrolysis and methyl- or ethyl-cyanoacrylates produce toxic break down products such as formaldehyde as well as eliciting strong inflammatory reactions. Because of this, methyl- and ethyl-cyanoacrylates are not used for biological purposes.[34]

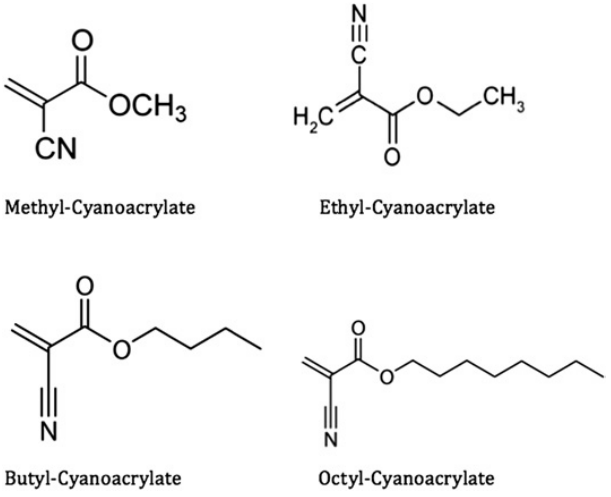

Figure i. Chemical formula of various types of cyanoacrylate monomers. Increasing side chain length decreases polymerization rate and tissue toxicity. Adapted from Kyle et al.

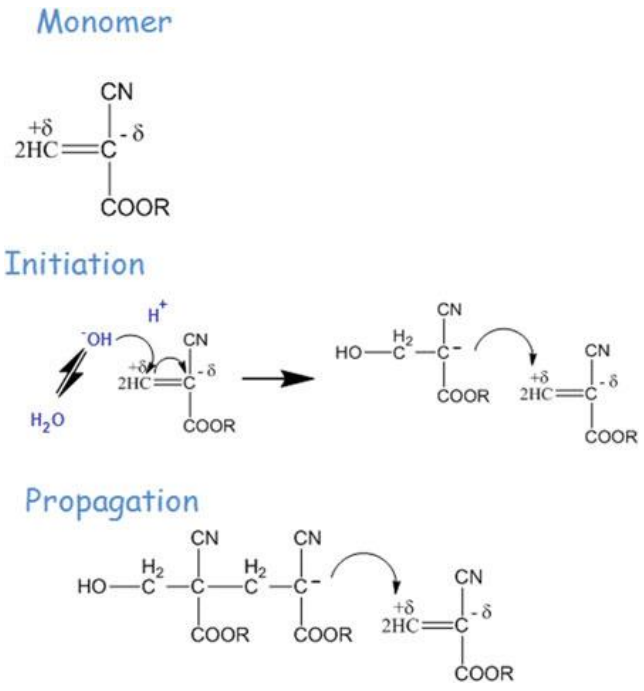

Figure ii. Polymerization mechanism of cyanoacrylate monomers via the anionic pathway. The presence of an anion such as OH^- initiates the reaction and results in chain polymerization.

Polymerization of cyanoacrylate monomers may occur via one of three mechanisms: anionic, zwitterionic or free radical. Figure ii. shows the polymerization mechanism of the anionic pathway. Of the three, the first two are strongly favored in-vivo due to the presence of hydroxide or amine groups in the body to initiate the polymerization reaction.[34, 36] These groups also serve as binding sites on proteins that allow strong bonds to form with cyanoacrylates.[37]

NBCA has a rapid polymerization time and good tissue adherence, making it an effective agent for sealing off abnormal vessel connections. However, this very property also makes it difficult to control as described in the previous section. Additionally, the catheter tip may become glued to the vessel wall, forcing the physician to leave a piece of the catheter behind. The ideal situation is for NBCA to polymerize in the AVM nidus itself, thereby arresting all flow into the AVM.

Pure NBCA reacts extremely fast in-vivo and is almost never used except in pediatric Vein of Galen malformations.[18] To decrease NBCA polymerization rate, various compounds can be added to create glue mixtures. Acetic acid has been used as a polymerization retardant to slow down the reaction mainly by decreasing the pH and the availability of anions such as OH^-. Currently, most centers use Lipiodol (ethiodized poppyseed oil) as both a radio-opaque agent and as a polymerization retardant. However, the addition of lipiodol to NBCA slightly raises the mixture's viscosity, requiring greater injection force by the operator.[38] In the earlier years, tantalum powder was added as a radio-opaque agent to allow visualization under fluoroscopy. However, some authors reported spontaneous polymerization of NBCA-tantalum mixture when it was left unused for 15 minutes or more.[31]

Typical mixtures of Lipiodol/NBCA encountered clinically are 50/50, 60/40, 70/30 (volume/volume). Increasing the amount of lipidol increases the polymerization time and conversely decreases the polymerization rate. However, choosing the right formulation, injection rate and glue volume is an imprecise science. To date, studies looking at the behavior of NBCA under physiological conditions have been rather crude. Most interventionalists rely on experience and look at the cerebral angiogram for clues. They determine the NBCA formulation based on nidus size, rate of contrast clearance, number of feeding arteries and draining veins. The injection rate is a bit more difficult to quantify, and most of the time it is an experienced "best guess". In the full column technique, once the glue is injected the catheter is quickly withdrawn to prevent adhesion of the tip to the vessel wall.

For comparison, the properties of Onyx will be briefly outlined below but will not be the focus of this study. Onyx consists of a suspension of EVOH polymer in dimethyl sulfoxide (DMSO) with tantalum powder added for radio-opacity.[18] Figure iii. below shows the chemical formula of Onyx. It should be noted that the polymer chains in Onyx suspension are already formed. Polymerization does not actually occur since the suspension does not contain monomer units. Instead, upon injection, DMSO quickly diffuses out of the suspension and EVOH precipitates into a soft rubbery mass in an aqueous environment, resulting in vessel occlusion. The occlusion time measured during injection reflects precipitation time rather than polymerization time. Onyx injections occur over the timespan of minutes and may extend up to half-hour or more. In fact, a slow injection rate is encouraged since prior animal work demonstrated that an overly vigorous injection can results in vessel tissue dissolution by DMSO with potentially catastrophic consequences. Time is needed for DMSO to dissolve and dilute into the bloodstream and surrounding interstitial tissue. Due to its slower rate of action compared to NBCA, complications can arise in high-flow AVMs where Onyx embolizes to the lungs. Although not an adhesive polymer, Onyx can also cause mechanical interference with the microcatheter, resulting in difficult removal similar to NBCA.

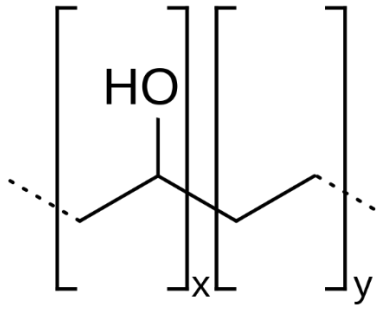

Figure iii. Chemical formula of Onyx, with variable (x) number of vinyl alcohol groups and (y) number of ethyl groups. The polymer chains dissolve well in DMSO and remain in suspension. In an aqueous environment such as blood, the polymer chains are relatively hydrophobic and interact with each other strongly (via hydrogen bond and van de Waals' forces), precipitating out of solution to form a soft rubbery mass.

Characteristics and factors that influence embolization – a brief analysis:

Characteristics: during embolization in the angiography suite there are several observable characteristics intrinsic to the AVM which can be summarized as morphologic versus hemodynamic (Table i).

Table i. Morphologic and hemodynamic characteristics that influence embolization

Morphologic	Hemodynamic
- AVM nidus size - Number of feeders/draining veins - Size of feeders/draining veins - Physical location of nidus, feeders, and draining veins (e.g. supra- vs. infratentorial, superficial vs. deep) - Nidus morphology: fistulous, plexiform, mixed, single vs multiple compartments, etc. - Presence of venous varices/ectasias - Presence of nidal/prenidal aneurysms	Non-invasive - Flow rate: estimated with frame-counting (at ≥4fps) or TCD - Regional variability in flow rate in nidus/feeders/draining veins (angiography or TCD) Invasive - Feeder/vein pressure - Feeder flow velocity (direct Doppler insonation)

The morphologic characteristics tend to have an association with the likelihood of complete nidus occlusion and complication rate as described in the previous section. The hemodynamic characteristics predominantly provide indirect flow rate information. Together with morphologic characteristics, they provide an indication of how quickly the glue needs to harden. Pressure measurements may provide an indication of the risk of hemorrhage with partial embolization or early vein occlusion, although the association is even less clear than the association with risk of spontaneous hemorrhage.[12] Intravascular pressures are also observed to rise as the feeder and its supplied nidus approach complete occlusion.[39]

Factors: In the angiography suite there appear to be a few modifiable factors that surgeons or neuro-interventionalists can control during the embolization of AVMs. These include:

- The distance between the microcatheter tip and the AVM nidus
- Glue mixture

- The power of the injection

How each of these factors influence embolization will be outlined below.

Distance between microcatheter tip and nidus

The longer the distance between the microcatheter tip and the nidus, the longer the time the glue needs to remain liquid. This information will need to be considered together with the estimated flow velocity (typically observed using frame-counting) to provide a time estimate. The location of the catheter tip may vary based on the injection technique. For example, a wedge technique would require the catheter tip to be within the nidus and "wedged" against the wall. In this case, the distance is essentially zero. In the full-column technique, the catheter tip will be parked in the feeder a short distance away from the nidus. In addition to technique, there are often limitations as to how far the catheter can be navigated. A very tortuous and/or distal feeder (e.g. distal anterior cerebral artery) will limit how close the microcatheter tip can be advanced to the nidus.

Glue mixture

Changes in the NBCA/Lipiodol mixture changes the rate of polymerization. Interventionalists alter this ratio based on how much time it is determined that the glue must remain liquid. This is described in the paragraph above. The ultimate goal is to have as much of the glue polymerize within the nidus as possible without premature occlusion of the feeding artery or draining vein. Currently, there is no good data that accurately reflects the in-vivo polymerization time of various NBCA/Lipiodol mixtures to help guide interventionalists. One of the objectives of this project is to generate such data to aid interventionalists during embolization.

Since polymerization is a continuous process, polymerization times should be calculated for each glue concentration at three different time points: t_0, t_{50} and t_c. t_0 is the time to onset of polymerization, first observed when the surface of the glue droplet opacifies and begins to deform. t_{50} is the time to achieve a 50% change in height of the glue droplet (or 50% reduction in diameter in any direction).

t_c is the time to complete polymerization (or over 95% polymerization based on continuous video observation). Distinction between the three time points is important because NBCA probably does not need to reach t_c for a significant change in AVM feeder to occur; it only need to be firm enough to occlude the feeder.

Injection power

This factor includes a number of components. Power = Work/Time and Work = Force x Distance. Looking at the relationship between a glue syringe and the interventionalist's hand, force is applied to the plunger which makes it travel a certain distance. This distance is directly related to the volume of glue injected. The amount of force needed to do this will depend on the total resistance of the syringe-microcatheter combination, the flow rate, as well as any additional downstream resistance from feeding artery pressure or partially formed glue casts. The time it takes to perform the injection will depend on both the injection velocity (how quickly the glue is pushed into the nidus) and the total volume of glue used (more glue = more time pushing the syringe). Injection power captures all of these variables into a single value, potentially allowing for objective quantification of operator-dependent injection parameters.

Taking a closer look at the amount of force exerted, the syringe in the operator's hand can be pushed with a certain amount of force. The greater the force, the higher the flow rate. The overall resistance of the system can be simplified to three factors:

1. Resistance from the syringe (different for 1cc vs 3cc)
2. Microcatheter Pouiselle flow resistance (see formula below)
3. Additional resistance from connectors and geometry changes

The size of the syringe has a direct effect on pressure (P) in the catheter since P = F/A, and for a given force (F) applied by the operator, a smaller syringe will have a smaller cross-sectional area (A), resulting in a greater pressure. This pressure will then cause greater volumetric flow rate (Q) based on the Pouiselle equation:

$$\Delta P = \frac{8\mu L}{\pi r^4} Q$$

Eq.1 *Pouiselle flow equation: L=length of catheter, μ=dynamic viscosity, r=radius of catheter*

If we examine the system in series the resistance acts in series. Starting with the applied pressure P_a by the operator, there will be a pressure drop across the plunger due to plunger friction ΔP_p. Next, there will be a pressure drop across the chamber of the syringe due to fluid resistance ΔP_s, which is probably negligible but will be included in the initial analysis and expressed as a loss coefficient C_s. There will be a pressure drop across the syringe opening due to orifice effect (vena contracta, an example is shown below in Figure iv.) ΔP_o, which can be presented as a loss coefficient C_o.

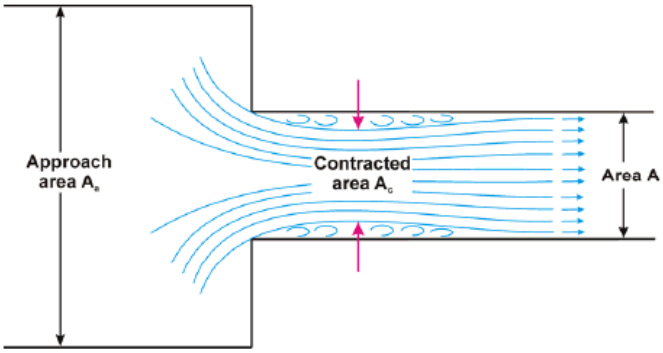

Figure iv. Example of vena contracta

There will be a pressure drop across the connector hub of the microcatheter ΔP_c, which can also be represented as a loss coefficient C_c. The pressure drop within the microcatheter ΔP_m is outlined below and the final outlet pressure at the very tip of the microcatheter is P_f. If we add the pressure drops all together, assuming the syringe is pushed slowly and the velocity inside is negligible, the total pressure drop across the system can expressed as:

$$\Delta P_{sys} = P_a - P_f$$

$$\Delta P_{sys} = \Delta P_p + \Delta P_s + \Delta P_o + \Delta P_c + \Delta P_m$$

Eq.2 *Overall pressure drop across the system*

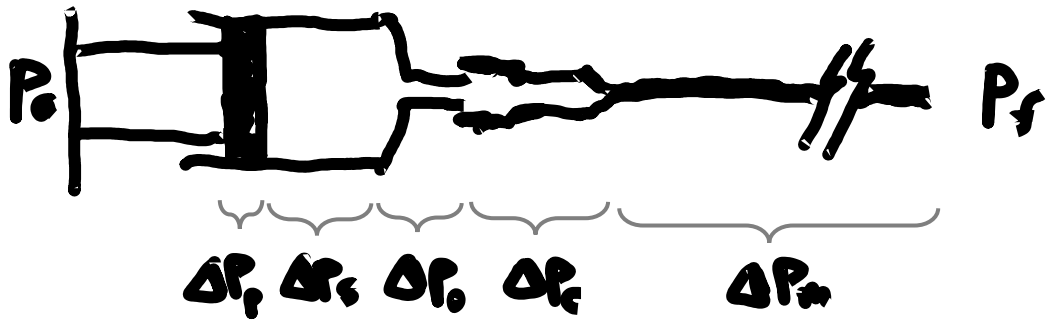

Figure v. Simplified diagram of the syringe/microcatheter system with labels of the pressure changes expected to occur at each subsection of the system.

For ΔP_p if we imagine the plunger as a cylinder with radius r_p and thickness t the pressure exerted by the friction force of the plunger rubber against the syringe wall can be calculated as follows:

$$\Delta P_p = \frac{f_* F_n}{\pi {r_p}^2 t}$$

Eq.3 *Pressure loss due to friction of the rubber plunger against syringe wall, equation variables are outlined below.*

Where f_* is the coefficient of friction (either static or kinetic depending on the plunger motion), and F_n is the radial normal force exerted by the plunger against the syringe wall. The coefficient of friction will change depending on whether the injection is just starting where f_s should be used versus if the injection is under way where f_k should be used instead. Practically speaking, it might be difficult to ascertain f_* ands F_n accurately, so the final value of ΔP_p will probably need to be measured empirically.

The remaining pressure drop terms can be expressed as:

$$\Delta P_s = C_s Q$$
$$\Delta P_o = C_o Q$$
$$\Delta P_c = C_c Q$$
$$\Delta P_m = \frac{8\mu L}{\pi r^4} Q$$

Eq.4 *Pressure drop terms for each subsection of the system*

Substituting the terms in equation 2 with equations 3 and 4 reveal the following equation:

$$\Delta P_{sys} = \frac{f_* F_n}{\pi {r_p}^2 t} + C_s Q + C_o Q + C_c Q + \frac{8\mu L}{\pi r^4} Q$$

$$P_a = \frac{f_* F_n}{\pi {r_p}^2 t} + C_s Q + C_o Q + C_c Q + \frac{8\mu L}{\pi r^4} Q + P_f$$

$$\frac{F_a}{A} = \frac{f_* F_n}{\pi {r_p}^2 t} + C_s Q + C_o Q + C_c Q + \frac{8\mu L}{\pi r^4} Q + P_f$$

Eq.5 *Pressure drop across the whole system expressed in detailed terms.*

There is the additional possibility to further simplify the equation by combining ΔP_p, ΔP_s and ΔP_o into a combined ΔP_{syr} as a pressure drop across the syringe and measure a combined loss coefficient C_{syr}. C_{syr} would be best obtained empirically by working backwards and measuring the minimum force needed to move the plunger in a filled syringe open to the atmosphere. An example of such a setup could use a modified Harvard syringe pump with a load cell connected in series to measure the force exerted by the pump at a certain plunger velocity. Substituting for this, we have:

$$\frac{F_a}{A} = C_{syr} Q + C_c Q + \frac{8\mu L}{\pi r^4} Q + P_f$$

Eq.6 *Simplified system pressure drop expressed in terms of applied force by the interventionalist.*

In the event that the flow deviates from ideal assumptions (i.e. non-laminar flow, can be checked by calculating the Reynold's number $\frac{\rho v L}{\mu}$), the more generalized Darcy-Weisbach equation can be used instead of Pousielle equation. If we were to also take into account the fluid velocity within the syringe then the application of Bernoulli's equation and continuity equation would be more appropriate and the loss terms will then depend on Q^2 instead of Q. Doing so would yield the following, assuming effect of gravity is negligible:

$$\frac{P_a - \Delta P_p}{\rho g} + \frac{V_0^2}{2g} = \frac{P_1}{\rho g} + \frac{V_1^2}{2g} + \left(f \frac{L}{D} + \Sigma K \right) \frac{V_1^2}{2g}$$

Eq.7 *Darcy-Weisbach equation for calculating system pressure drop for non-ideal flow: f =Darcy friction factor, L=length of microcatheter, D=diameter of microcatheter, K=loss coefficients at the nozzle and connector.*

The pressure inside the syringe is expressed by the $P_a - \Delta P_p$ and the average velocity of the fluid moving inside the syringe is V_0. P_1 and V_1 denotes the pressure and velocity at the end of the microcatheter right before the fluid exits into the blood stream. K is the aggregate loss coefficient from the two step-down contractions at the syringe nozzle and at the converging connector (confuser geometry) to the microcatheter. It is well known from experimental studies that pressure losses at contractions are dependent on downstream velocity only (i.e. V_1). The average velocities can be converted to volumetric flow rates (Q) based on the simple equation Q = VA, where V is the average velocity and A is the cross-sectional area. For Q_0 the area of the syringe plunger is used and for Q_1, the cross-sectional area of the inner lumen of the microcatheter is used.

Finally, it is worth noting that the exit condition in the blood stream is dependent on the difference in velocity between the free stream and the catheter glue flow as well the difference in viscosity between the two mediums. This can be best described as a co-axial flow scenario where the outer shell flow consists of blood and the inner stream injected into this shell flow (also known as bulk flow) consists of NBCA/Lipiodol mixture. Idealized flow studies have shown an important transition from dripping to jetting flow depending on the difference in velocity and viscosity.[40] In the real-world scenario, wall interaction and polymerization (transformation from liquid to solid) during injection further complicates the analysis.

The analysis above serves as a conceptual framework when considering the variables involved in the injection of liquid embolic agents during treatment of brain AVMs. In the present study, the interaction between glue, vessel wall and bulk flow will be characterized via simulated in-vivo injections. The remaining parts of the aforementioned analysis will not be explored due to scope and time constraints. The analysis lays the foundation for future work and will be part of another study aimed at quantifying the injection parameters during brain AVM embolization.

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Integrated Article: Characterization of NBCA – Manuscript Draft

Target Journal: Journal of NeuroInterventional Surgery (JNIS)

Title: A systematic characterization of the factors influencing polymerization and dynamic behavior of n-butyl cyanoacrylate (NBCA)

Bill H. Wang[1], David M. Pelz[1,2], Melfort R. Boulton[1,2], Donald H. Lee[1,2], Stephen P. Lownie[1,2]

1. Department of Clinical Neurological Sciences, London Health Sciences Centre
2. Department of Medical Imaging, London Health Sciences Centre

Background and objective:

Brain arteriovenous malformations (AVMs) are abnormal connections between arteries and veins without an intervening capillary bed.[1, 2] Their prevalence ranges from 1 to 20 per 100,000 patients, and of these, approximately half will present with an intracerebral hemorrhage resulting in significant morbidity (30-40% per event) and mortality (10% per event).[1-6] Endovascular glue embolization with injection of N-butyl cyanoacrylate (NBCA) is an accepted form of treatment.[7-10] It is most often used as an adjunct to surgery or radiosurgery, although it can be sufficient as a standalone treatment in approximately 5-10% of cases.[8] In carefully selected cases the cure rate with endovascular embolization can be higher, ranging from 20% to 50%.[7, 11-15]

Although ethylene vinyl alcohol copolymer (EVOH, sold by Covidien as Onyx®) has quickly become a preferred liquid embolic agent at many centers, there are certain high-flow AVMs (usually with fistulous components) where Onyx cannot be used due to the risk of passage into the venous outflow system. This typically results from inadequately fast polymer precipitation rate to match the AVM flow rate. NBCA remains a mainstay of treatment in such situations but is also associated with complications. Most of the complications that occur during embolization with NBCA are related to polymerization rate.[7, 8] A rate that is too fast may cause proximal feeding artery occlusion. Glue could then reflux back into normal upstream vessels, causing stroke. Conversely, a rate that is too slow could cause the glue to occlude the draining vein, or embolize to dural sinuses and the lung.[16-

18] Premature venous drainage occlusion may raise the AVM intranidal pressure and trigger intracranial hemorrhage. [19-21]

Existing scientific literature reports a wide range of polymerization rates with significant discrepancies between in-vivo and in-vitro results.[8, 22, 23] Furthermore, the interaction between glue and vessel surface, as well as the presence of biologic catalysts are poorly understood. Therefore, despite its clinical use for over 4 decades (FDA approved in 2000 with use dating almost 3 decades prior)[9, 10], there is still incomplete understanding of NBCA polymerization and injection behavior.[8] This has resulted in wide variation with respect to the degree of dilution (typically modified by altering glue/Lipiodol or glue/acetic-acid ratio)[24] and injection technique (sandwich, wedge or full-column technique) used for NBCA embolization procedures.[7, 8]

The decreasing amount of NBCA operator experience with the introduction of Onyx coupled with the incomplete understanding of NBCA behavior further exacerbates treatment risk for patients, especially in situations where NBCA is the only option. In this study, we aim to systematically characterize the factors that influence NBCA polymerization in both static and dynamic environments. Such information could help interventionalists to optimize injection parameters and potentially reduce complications.

Methods:

From previous studies[8, 22-25] and our preliminary data, NBCA appears to behave differently in the presence of biological tissue compared to tissue-free vessel substitutes. NBCA interactions were deconstructed into three categories: influence of physical parameters (namely temperature and pH); physical interaction between NBCA and vessel wall (both non-biological and biological); and influence of exposure to biological tissue.

The influence of pH and temperature on NBCA polymerization time was measured. The submerged droplet test method was employed where small droplets of NBCA (~0.1cc) were deposited on an

inert PVA-C surface (confirmed through preliminary work) and continuously monitored via high-definition video recording system. The glue was kept submerged in an aqueous environment to simulate the physical conditions during embolization. Polymerization time was measured for clinically relevant mixtures of Lipiodol/NBCA of 50/50, 60/40, 70/30 ratios (NBCA was mixed with Lipiodol to provide radio-opacity and to delay polymerization). Polymerization times were recorded for solution pH values of 4, 5.5, 6.5 and 7.4 at temperature of 37.4°C. This corresponded to physiologic solutions of D5W, normal saline, Ringer's lactate, and Plasma-Lyte® respectively. These solutions provided an aqueous acellular and plasma-free environment. Polymerization times were also recorded for solution temperature values of 15, 20, 25, 30, 35 and 37.5°C at pH of 7.4 (Plasma-Lyte® solution used).

The physical interaction between NBCA and the vessel wall was analyzed using the contact angle method with the important modification of performing all measurements in a submerged aqueous environment. Native avian endothelium was used as the reference surface and droplets of NBCA (~0.1cc) were carefully placed on the flattened vessel surface using a syringe with a 25-gauge needle. Silicone (both plain and heparin coated) and poly-vinyl alcohol cryogel (PVA-C) surfaces were tested as vessel substitutes and compared to the endothelial surface. Silicone was chosen due to its ubiquity and its status as a "de facto" vessel substitute in numerous prior studies involving NBCA. PVA-C was chosen as a hydrophilic material that better replicates the physical properties of an endothelial surface. All tests were carried out under physiologic conditions (pH 7.4, temperature 37.5°C) in an acellular and plasma-free environment.

To delineate the role of potential biological catalysts, the endovascular environment was separated into endothelial surface and blood products. The effect of endothelial surface was assessed by placing NBCA droplets on a flattened viable (<6 hours from time of harvest) endothelial surface while video-recording the polymerization reaction. The effect of blood products was further broken down into plasma, platelets, packed red blood cells (RBCs), and lysed RBCs. Polymerization times were

measured by first placing a layer of each of the respective blood products on a PVA-C (inert) surface, followed by placing NBCA droplets onto the surface. All tests were performed in an aqueous environment at pH of 7.4 and temperature of 37.5°C. Since polymerization is a continuous process, polymerization times were calculated for each glue concentration at three different time points: $\mathbf{t_0}$, $\mathbf{t_{50}}$ and $\mathbf{t_c}$. $\mathbf{t_0}$ is the time from initial glue deposition to the onset of polymerization, first observed when the surface of the glue droplet opacifies and the droplet just begins to deform. $\mathbf{t_{50}}$ is the time to achieve a 50% change in height of the glue droplet (or 50% reduction in diameter in any direction). $\mathbf{t_c}$ is the time to near-complete polymerization (or over 95% polymerization based on continuous video observation).

Visual assessment of polymerization was performed based on video recording footages. Frame-by-frame analysis (video recorded at standard 30fps) was performed to accurately calculate $\mathbf{t_0}$, $\mathbf{t_{50}}$ and $\mathbf{t_c}$ times. To further confirm that near-complete polymerization had truly occurred at $\mathbf{t_c}$, we performed indentation tests on select glue droplets using an ASTM type OO indenter with a modified 10-gram load. This allowed us to assess whether the center of the glue droplet still remained liquid once the outer shell had opacified.

Finally, to examine the dynamic behavior of NBCA/Lipiodol mixture in an AVM feeding artery during microcatheter injection, high-speed video analysis of glue injection was performed to characterize flow and simulate in-vivo conditions. A flow circuit was constructed using a pulsatile pump with tubing connected to straight vessel substitutes made of heparin coated silicone (Medtronic, USA). Heparin coating improves surface hydrophilicity compared to plain silicone while providing transparency for high-speed video recording. A flow bypass circuit was connected parallel to the vessel substitute. This allowed the pressure and flow rates to be adjusted to match conditions typically encountered in an AVM feeder. A rotating hemostatic valve (RHV) was connected upstream of the vessel substitute to allow microcatheter insertion (SL-10). The dynamic behavior of

NBCA/Lipiodol flow within the vessel was considered akin to co-axial flow as described by Utada et al, with a transition from "dripping" to "jetting" as the outer shell (blood) flow increased.[26]

At least 3 droplets were used for each glue formulation/test condition combination. Statistical significance was determined using a two-tailed Student's t-test, ANOVA or chi-squared analysis wherever appropriate. Results are expressed as mean +/- Standard Deviation (SD). Results are considered significant when $P < 0.05$.

Results:

pH and Temperature: The polymerization times for three different formulations of Lipiodol/NBCA (referred from here on in as "glue" for simplicity) are plotted against pH and temperature in Figures 1A and 1B respectively. Polymerization time decreased (indicating increasing polymerization rate) with increasing pH as well as increasing NBCA glue concentration. Both pH and glue formulation independently influenced polymerization time and their effects are statistically significant based on 2-factor ANOVA. The influence of temperature on polymerization rate is plotted in Figure 1B. Although the brain is at body temperature during interventional procedures, the catheters are constantly flushed with ambient temperature solution. As such, the AVM nidus temperature could actually be slightly below body temperature. As expected from reaction kinetics, increasing

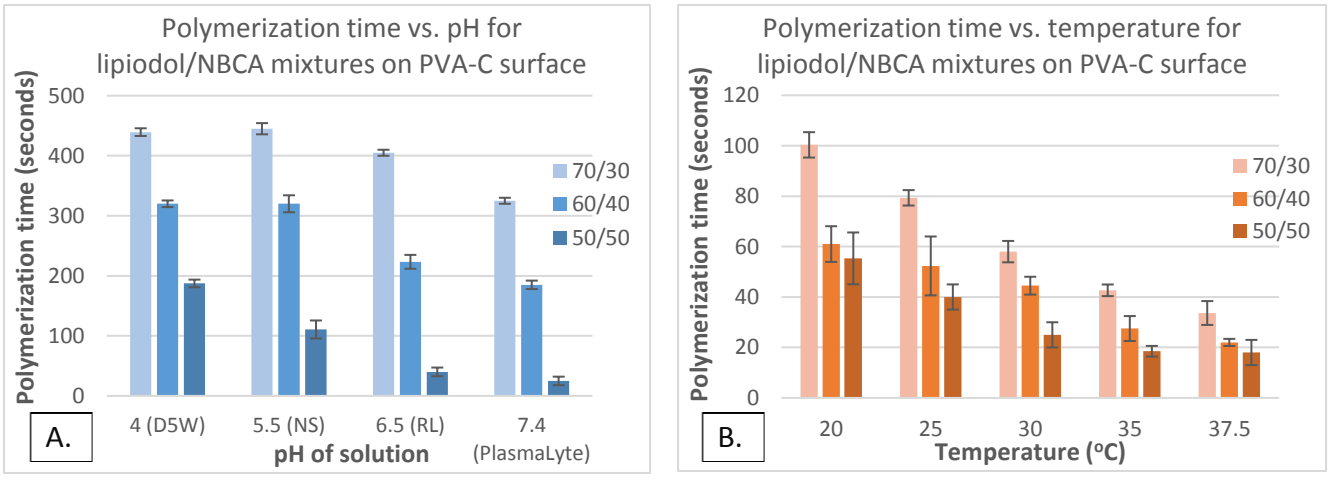


Figure 1. Polymerization time vs pH (A) and temperature (B) plots for three different mixtures of lipiodol/NBCA

temperature caused a corresponding increase in polymerization rate. Both temperature and glue formulation also independently influenced polymerization time with statistical significance (2-factor ANOVA).

NBCA – Vessel Wall interactions: The submerged contact angle of glue droplets on silicone (52±3°, Figure 2A), heparin coated silicone (84±3°, Figure 2B) and PVA-C (150±8°, Figure 2C) were markedly different from each other (pairwise comparisons using two-tailed Student's t-test all resulted in $p<0.05$). The contact angle between glue and endothelium was found to be 151±3° (Figure 2D). There was no statistically significant difference in contact angle between endothelial and PVA-C surfaces. However, the difference between the endothelial and the two silicone surfaces were significantly different (Figure 3). There was no statistically significant difference in contact angle for a given surface between various glue formulations (50/50, 60/40 and 70/30, data not shown).

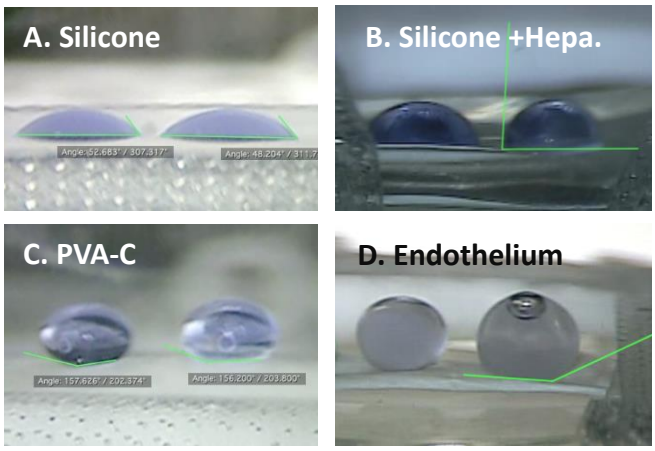


Figure 2. Contact angle measurements between NBCA/Lipiodol and difference surfaces submerged in D5W at 37.5°C: silicone, silicone with heparin coating PVA-C and endothelium surfaces

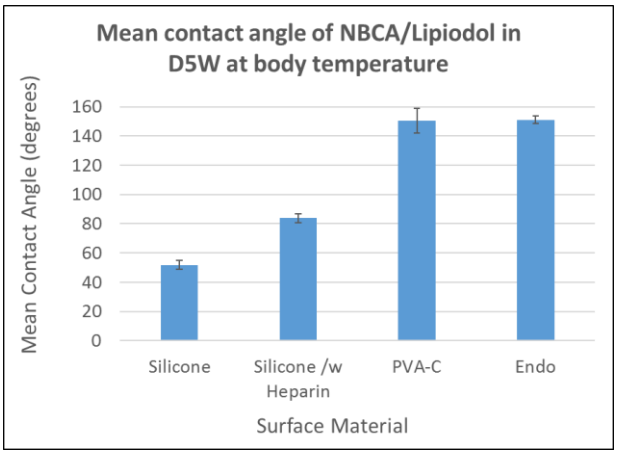


Figure 3. Contact angle measurements for silicone, silicone with heparin coating, PVA-C and endothelium surfaces

Biological Catalysts: From previous studies[8, 22-25] and our own observations, glue polymerization rate increases in the presence of biological tissue. Figures 4 shows time-lapse photographs of glue droplets (60/40 formulation) on an endothelial surface (pH=7.4, 37.5°C). Figure 5 shows glue droplets (60/40 formulation) on a PVA-C surface at 5 minutes after initial placement and

confirmatory indentation test to assess extent of polymerization. Glue droplets on both surfaces started out at t=0 seconds looking identical with similar appearances to those shown in Figure 2C and 2D. However, as time progressed the glue droplets on the endothelial surface started polymerizing from the bottom-up. This directional preference caused the droplet to flatten out over time with complete polymerization occurring at approximately t=90 seconds. In contrast to this, the glue droplets on the PVA-C surface (Figure 5) opacified and flattened somewhat initially but then changed very little as time went on. Indentation test was performed at t=300 seconds on glue droplets on the PVA-C surface. Despite the glue droplets' optical appearance, their centers never truly polymerized even after 300 seconds. This was demonstrated by the blue liquid glue streak running between the polymerized shell and the type OO indentor tip.

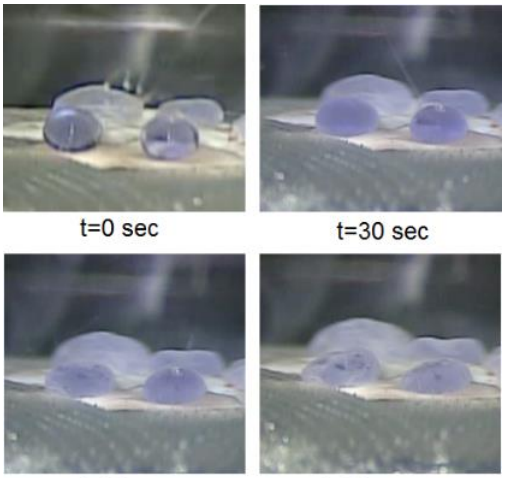


Figure 4. Endothelium catalyzes polymerization from the bottom-up, effectively flattening the droplet as polymerization approach t_c of approximately 90 seconds

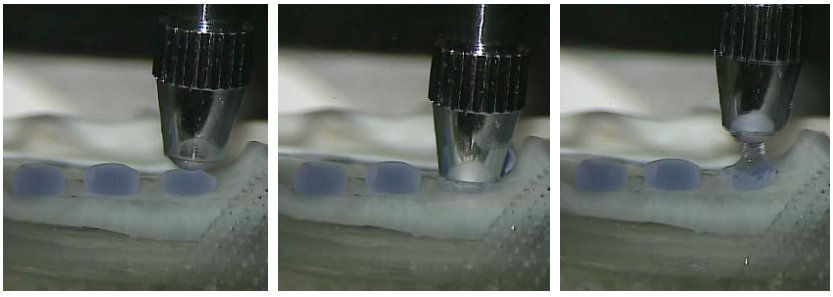


Figure 5. Indentation test on PVA-C surface at t=300sec (60/50 pH 7.4) showing incomplete polymerization with liquid centre

The decomposition of the endovascular environment yielded the following major biological components: endothelium, plasma, platelet and RBC. Their effect on glue polymerization time was further explored. Given that polymerization is a continuous process, the times $\mathbf{t_o}$, $\mathbf{t_{50}}$ and $\mathbf{t_c}$ were calculated for each glue formulation and biological substrate combination as shown in Figures 6-8.

Table 1 shows the polymerization times for glue droplets on an endothelial surface. Overall, polymerization times decreased as the biological substrate varied from endothelium, plasma, platelets, packed RBC and lysed RBC. Lysed RBC appear to result in the greatest decrease in polymerization time (corresponding to the greatest increase in polymerization rate) as demonstrated based on the measured values of $\mathbf{t_o}$, $\mathbf{t_{50}}$ and $\mathbf{t_c}$.

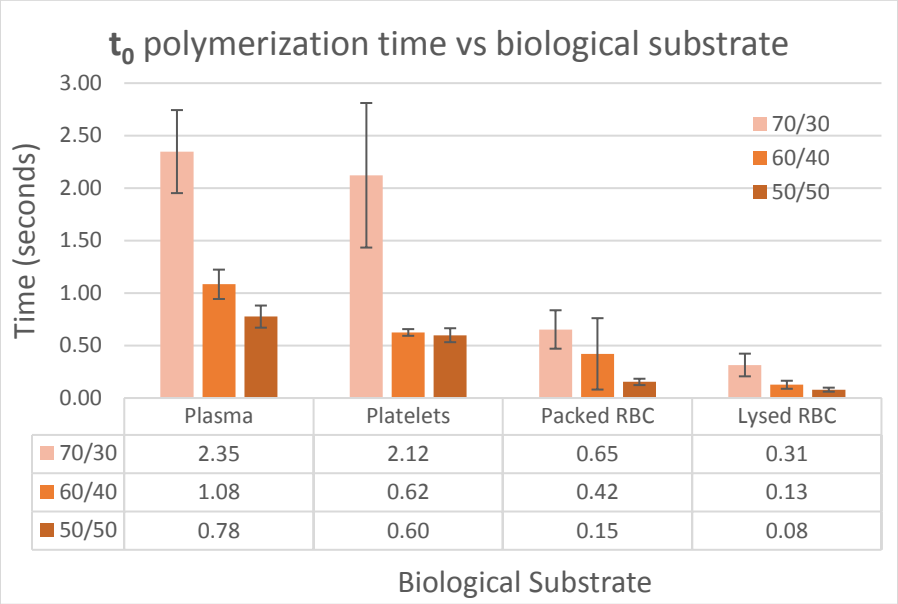


Figure 6. The effect of plasma, platelets, packed RBC and lysed RBC on the time to onset of polymerization $\mathbf{t_0}$ for various glue formulations

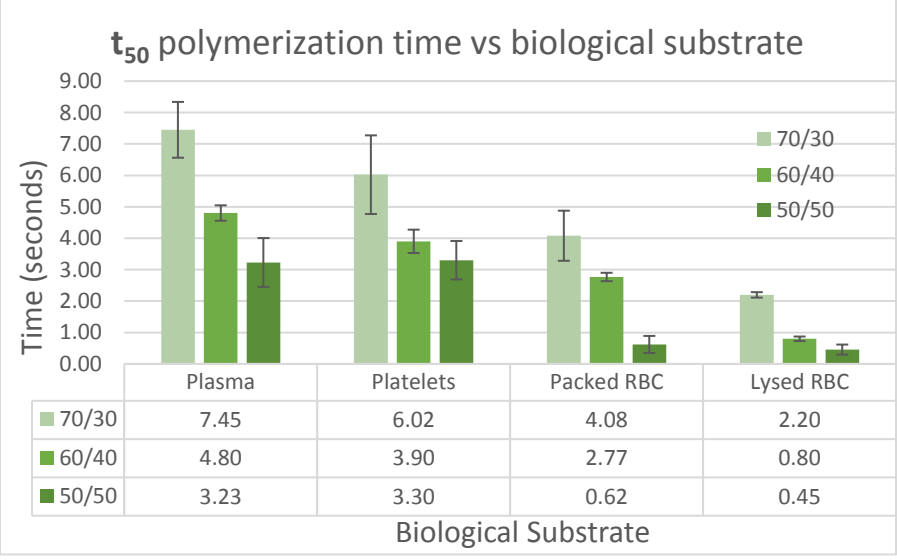


Figure 7. The effect of plasma, platelets, packed RBC and lysed RBC on the time to achieve 50% change in dimension of glue droplet $\mathbf{t_{50}}$ for various glue formulations

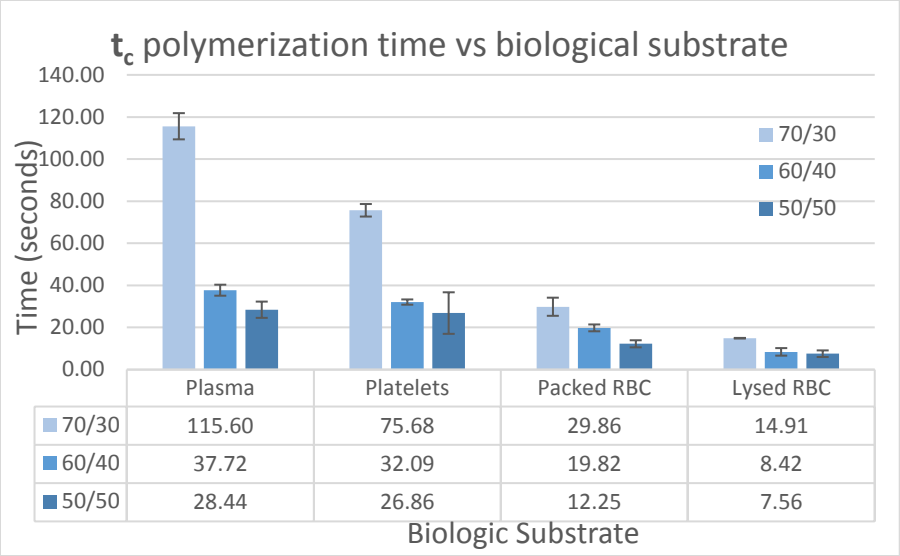


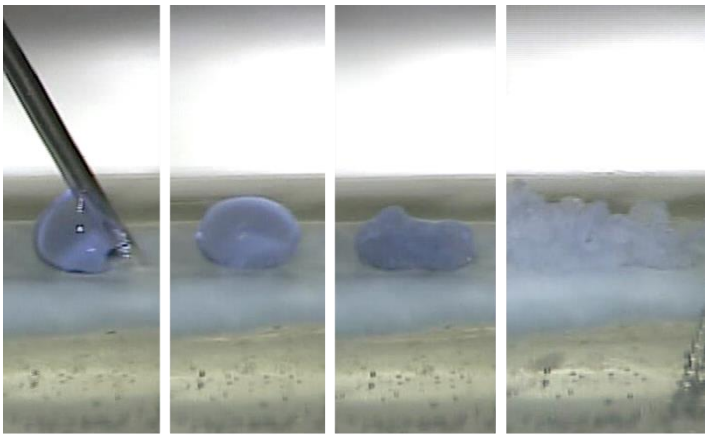
Figure 8. The effect of plasma, platelets, packed RBC and lysed RBC on the time to achieve near-complete (>95%) polymerization t_c for various glue formulations

Table 1. Effect of endothelium (no blood products) on polymerization times t_0, t_{50} and t_c for various glue formulations, measured in seconds

	Glue: 50/50	*Glue: 60/40*	*Glue: 70/30*
t_0	0.6 ± 0.3	1.6 ± 0.4	13 ± 3
t_{50}	8 ± 2	72 ± 6	614 ± 31
t_c	98 ± 4	416 ± 6	1880 ± 68

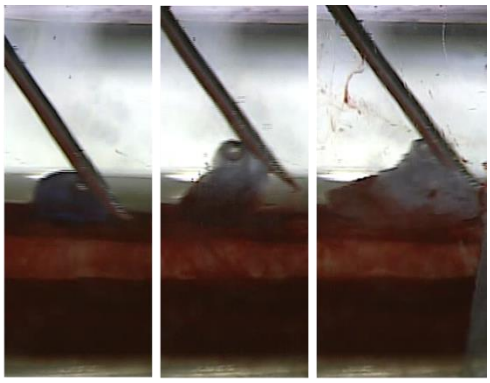
Figure 9 shows an example of polymerization time measurements for 70/30 glue formulation upon exposure to plasma. The glue appeared transparent upon initial deposition at t = 0 seconds. At t_0 = 2.4 seconds the glue droplet opacified and began to deform, providing a visual indication of the start of polymerization. At t_{50}=7.5 seconds the glue droplet flattened to approximate 50% of the original height and appeared more irregular due to ongoing polymerization. Finally, at t_c = 116 seconds polymerization was complete and the glue droplet lost its bluish hue while taking on an irregular multi-lobulated morphology. Figure 10 shows the exposure of 60/40 glue formulation to lysed RBC which resulted a very fast polymerization time. t_0 occurred almost instantaneously at 0.13 seconds and t_{50} was achieved at only 1 second after glue deposition. Complete polymerization occurred after approximately 8 seconds with the glue cast exhibiting a similar white irregular appearance as Figure

9. The polymerization time was so rapid that the NBCA droplet did not have sufficient time to detach from the depositing needle, polymerizing at the tip.



t=0 sec t_0=2.4 sec t_{50}= 7.5 sec t_c= 116 sec

Figure 9. t_0, t_{50}, and t_c times for 70/30 glue formulation during exposure to plasma



t_0=0.13 sec t_{50}= 1 sec t_c= 8 sec

Figure 10. t_0, t_{50}, and t_c times for 60/40 glue formulation during exposure to lysed RBCs

In Vivo Simulation: Figure 11 illustrates the idealized model of coaxial flow as previously described by Utada et al. The outside shell flow is akin to the blood flow in an AVM feeding artery while the central coaxial flow mimics the microcatheter glue injection. As the injection rate increased, there appeared to be a transition from dripping to jetting flow.[26] Figure 12 shows the experimental observations (recorded at 240fps), where three distinct phases are noted. Phase 1 (12A) consisted of dribble flow that lasted only 1-2 seconds before the whole microcatheter was filled with NBCA. Phase 2 (12B) continued as jetting flow (short jet) with length of jet dependent on outer shell flow velocity and vessel diameter. Phase 3 (7C) occurred when NBCA wetted the vessel wall and continued to roll off the vessel wall, sending droplets downstream so long as the glue remained liquid. Phase 3 was not described by Utada et al.[8] since their model assumed the injection catheter to be in the middle of the vessel, free from any wall-effect. Based on our observations, during endovascular microcatheter injections, it is more common to have the microcatheter tip against the wall or pointed at an angle towards the wall.

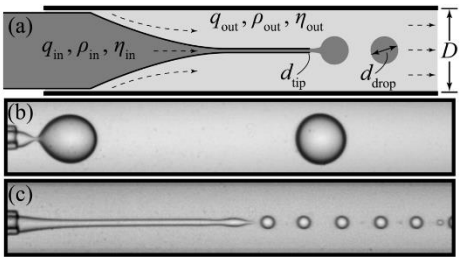


Figure 11. Coaxial flow

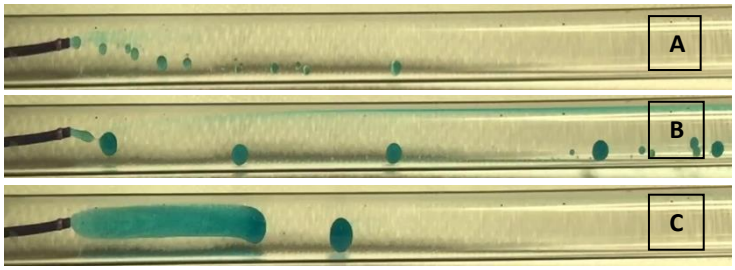


Figure 12. Screenshots of high speed video analysis of NBCA injection illustrating an initial period of dribbling flow (A, <1sec) followed by short jetting flow (B, 1-2 sec). Wall-effect eventually takes over (C) at variable time point depending on microcatheter position (typically starting at approximately 3 seconds).

Discussion:

In this study, we sought to systematically characterize the factors that influence NBCA polymerization in both static and dynamic environments. The effect of pH and temperature on glue polymerization time is known. However, a detailed quantification of their effect on glue polymerization has not been previously reported. Exposure to anions is the most common way to initiate polymerization in cyanoacrylates. Anions act as electron donors to initiate the reaction. Once initiation occurs, chain propagation will continue spontaneously for variable lengths.[27] The hydroxide (OH^-) anion is ubiquitous in aqueous environments, present whenever there is H_2O. In the clinical setting, prior to glue injection the microcatheter is thoroughly flushed with D5W (5% dextrose solution) with the common explanation that D5W does not contain any ions. From a basic chemistry stand point, this is false since all solutions will contain ions. It is the relative concentration of these ions that will influence polymerization time, and D5W happens to be the most acidic isotonic solution used clinically with a pH of 4. This translates to the lowest concentration of OH^- groups available of any solution, resulting in the best prevention against polymerization.

The physical interaction of glue with different surfaces provides some caution in interpreting in-vitro flow studies in existing scientific literature. Silicone is often used as a de-facto vessel substitute in flow models. However, the behavior of glue on silicone is quite different from that of endothelium.

Glue tends to spread more easily and stick to a silicone surface more readily, effectively "coating" it due to the shallow contact angle. This reduces glue flow velocity compared to the average bulk flow velocity and gives glue more time to polymerize in the silicone tubing. Therefore, studies employing silicone flow models to study glue embolization may over-estimate the effect of glue polymerization (especially in the absence of any biological tissue) in achieving flow arrest.[28]

As a mock vessel surface, PVA-C provided the closest mimicry of endothelium. However, due to the lack of any catalytic activity, it was paradoxically more difficulty to achieving flow arrest when injecting glue into PVA-C tubing. During polymerization, glue would form strong chemical bonds with the endothelial surface (based on our observations) that could act as anchor points. Glue did not bond to PVA-C readily, further exacerbating the flow arrest problem. In-vitro studies that employ mock vessels with hydrophilic surfaces should be interpreted in light of these findings.

The effects of biological substrates on glue polymerization time is significant and may help explain some of the discrepancies in previous studies that measured the polymerization time of NBCA in the in-vitro setting. In one of the earliest papers, Stoesslein et al. measured the in-vitro polymerization rate of NBCA/Lipiodol mixtures by bringing it into contact with citrated blood on a cover glass slip and visually observing polymerization.[24] The authors did not further specify the criteria used to judge polymerization, although the values that they published appeared closer to $\mathbf{t_c}$. Brothers et al. used a very different in-vitro method where glue droplets were dripped into human plasma held in a transparent cup with a piece of newspaper underneath it.[23] Opacification of the newspaper letters was defined as polymerization time, which is likely in between $\mathbf{t_0}$ and $\mathbf{t_{50}}$. Interestingly, both authors noted that in-vivo studies seemed to result in faster polymerization times compared to in-vitro results.

A few explanations were entertained to explain the in-vitro/in-vivo discrepancy, such as mixing caused by bulk flow of blood and the greater availability of anions in-vivo. In light of the results of

this study, it was fortuitous that Stoesslein et al. used whole blood as a biologic catalyst (albeit unknowingly). However, their values were closer to $\mathbf{t_c}$ (3.2, 4.7 and 7.5 seconds for 50/50, 33/66 and 25/75 formulations respectively) than $\mathbf{t_{50}}$ since they were measuring near-complete polymerization. Brothers et al. on the other hand used plasma, which would give a slower polymerization rate. However, they chose opacification as the polymerization end point which occurs quite early as seen in Figures 4, 5, 9 and 10. This provided partial offset to the slower polymerization time in plasma and the study ended up with values (0.7, 2.5, 3 and 4 seconds for 100/0, 50/50, 33/66 and 25/75 formulations respectively) that were closer to $\mathbf{t_{50}}$ in pRBC.

In the in-vivo setting, glue polymerization cannot be measured directly, and operators must use other clues such as nidus penetration or flow arrest as surrogate measures. This depends not only on polymerization time, but also on nidus morphology. Polymerization times may seem faster in-vivo for two reasons: 1. The presence of biologic substrates (especially RBCs) and 2. Interference from nidus or proximal feeders that allows partially polymerized glue to change/block flow before $\mathbf{t_c}$ is reached. It is for the second reason that we measured not only $\mathbf{t_c}$, but also $\mathbf{t_0}$ and $\mathbf{t_{50}}$**.** Looking at the values of $\mathbf{t_{50}}$, they appear to better approximate the time scales encountered clinically during embolization of AVMs (where contrast filling of the nidus can occur as quickly as 0.25 – 0.5 seconds). Glue does not need to fully polymerize to exert its effect, it just has to be hard enough to plug part of the nidus or feeding artery for stasis to occur.

Exposure to lysed RBC appears to result in the fastest glue polymerization time. The exact reason for this is unknown. It is known that amine groups from proteins can act as electron donors to initiate polymerization via a zwitterionic mechanism.[27] Cyanoacrylates have also been shown to form covalent bond with proteins (i.e. protein adducts) upon exposure to biologic tissue.[29] Cell membrane components and charge probably do not fully explain this since both endothelium and platelets contain cell membranes and neither substrate showed polymerization times that were as

rapid. Proteins and dissolved ions in general do not fully explain this either, since plasma contain abundant quantities of proteins (coagulation factors, immunoglobulins, albumin, etc.) and ions but does not have as rapid a polymerization time. Since lysed RBCs resulted in even faster polymerization rates than whole RBCs, this leads us to surmise that RBC-specific intracellular content plays the biggest role, and hemoglobin is highly suspect. More work will be needed to further delineate this.

High-speed video analysis of microcatheter glue injection revealed that unlike the idealized flow model, wall interaction is an important factor to consider. Wall interaction causes smearing and sticking of liquid glue with possible formation of anchor points after polymerization occurs. The heparin coated silicone surface provides good light transparency but does not provide the same degree of hydrophilicity as endothelium. The smearing will likely be less prominent in an endothelial surface. However, endothelium does have some degree of catalytic activity and also bonds to NBCA very well. Therefore, premature feeding artery occlusion is still a concern even with a well-adjusted glue formulation. A partially formed adherent glue cast in the proximal feeder (due to the wall-effect) could trigger more glue adhesion and polymerization, resulting in feeding artery occlusion before the nidus is fully embolized. One of the best ways to mitigate this problem is to navigate the microcatheter as close to the nidus as possible, with the wedge technique being an extreme example of this. Both the sandwich and full-column technique will be subject to wall-effect if the microcatheter tip is parked in the proximal feeding artery some distance away from the nidus.

Conclusion:

We have successfully deconstructed the dynamic behavior of NBCA embolization into its components to allow for better characterization of polymerization and injection parameters. PVA-C provides a better vessel substitute with respect to physical surface properties; however, it does not possess any catalytic activity encountered in the in-vivo setting. Biological catalysts play a substantial role in increasing polymerization rate with exposure to RBCs being the most important factor. Clinically

relevant polymerization times likely fall somewhere closer to $\mathbf{t_{50}}$ than to $\mathbf{t_c}$ which may help optimize glue formulation selection. Clinically, continuous flushing of the microcatheter with D5W provides an acellular/plasma-free environment with the lowest pH to prevent unanticipated early polymerization which is most likely related to contamination with RBC's. High-speed video analysis reveals that microcatheter injection exhibits dripping to jetting transition with an important additional phase of vessel wall interaction. A refined understanding of the polymerization behavior of NBCA could help reduce embolization-related complications.

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Appendix I

Onyx static droplet test

Droplet injection tests were performed for Onyx for comparison. The precipitation of Onyx appears to be very different from the polymerization of NBCA (Figure s1). Onyx has higher viscosity than Lipiodol/NBCA mixture and flows out of the syringe like a viscoelastic material rather than a liquid. The outer shell appears to precipitate first and solidify into a semi-soft solid within 1-2 seconds and no further outward indication of precipitation can be observed. Further injection results in liquid Onyx flowing between the formed cast and the vessel surface, with progressive widening of the base. Newly injected Onyx appear to adhere to existing precipitated Onyx. The center portion remains liquid for a significantly longer period of time and can be seen from the Onyx streaking upon withdraw of the 25-gauge syringe needle. There is no significant correlation between pH and precipitation rate. There is also no significant difference between silicone, PVA-C and endothelial surfaces. The presence of endothelium does not appear to increase the precipitation rate. The effect of blood on Onyx precipitation rate was not investigated.

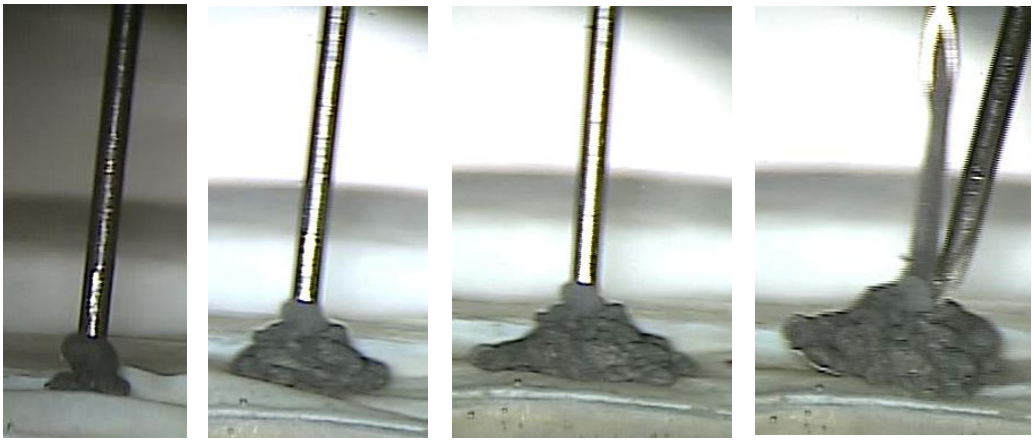

Figure s1. Injection of Onyx on endothelium (pH 7.4, temp 37.5°C). The method used for the assessment of NBCA polymerization time with $\mathbf{t_0}$, $\mathbf{t_{50}}$, and $\mathbf{t_c}$ cannot be applied here. The panel from left to right show injection times of 0, 3, 15 and 30 seconds respectively with injection been slowly and continuously performed for 15 seconds. No adhesion to endothelium was observe and no outward evidence of continuing precipitation can be appreciated. The center still appear liquid based on streaking from needle tip in the last frame.

Appendix II

Statistical Analysis

Polymerization time vs. temperature
Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
15C	3	208.97	69.65667	2434.973
20C	3	216.6	72.2	600.33
25C	3	171.63	57.21	404.7933
30C	3	127.5	42.5	275.25
35C	3	88.67	29.55667	149.2196
37.5C	3	73.67	24.55667	66.28963
50/50	6	186.1	31.01667	207.1257
60/40	6	262.3	43.71667	246.9817
70/30	6	438.64	73.10667	1229.367

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Temperature	6148.924	5	1229.785	5.421256	**0.011396**	3.325835
Glue formulation	5593.26	2	2796.63	12.32837	**0.002**	4.102821
Error	2268.45	10	226.845			
Total	14010.63	17				

Polymerization time vs. pH
Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
pH 4	3	946.666	315.5553	15890.82
pH 5.5	3	875.667	291.889	28537.31
pH 6.5	3	668.333	222.7777	33306.48
pH 7.4	3	535	178.3333	22533.33
50/50	4	363	90.75	5541.196
60/40	4	1048.333	262.0833	4717.37
70/30	4	1614.333	403.5833	3056.909

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Solution pH	35733.17	3	11911.06	16.96229	**0.002463**	4.757063
Glue formulation	196322.6	2	98161.32	139.7895	**9.27E-06**	5.143253
Error	4213.249	6	702.2081			
Total	236269.1	11				

T0 Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Endothelium	3	14.87033	4.956778	45.7889
Plasma	3	4.2065	1.402167	0.694168
Platelets	3	3.344833	1.114944	0.760792
Packed RBC	3	1.226833	0.408944	0.062517
Lysed RBC	3	0.520333	0.173444	0.015471
70/30	5	18.18683	3.637367	26.73539
60/40	5	3.807	0.7614	0.31687
50/50	5	2.175	0.435	0.091528

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Biologic	44.98624	4	11.24656	1.414908	**0.312643**	3.837853
Formulation	31.05479	2	15.52739	1.953472	**0.203779**	4.45897
Error	63.58891	8	7.948614			
Total	139.6299	14				

T50 Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Endothelium	3	693.861	231.287	110564.3
Plasma	3	15.4835	5.161167	4.551478
Platelets	3	13.22633	4.408778	2.044485

Packed RBC	3	7.466	2.488667	3.051579
Lysed RBC	3	3.452167	1.150722	0.851507
70/30	5	633.2078	126.6416	74062.98
60/40	5	84.474	16.8948	958.1489
50/50	5	15.80717	3.161433	9.804643

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Biologic	124774.7	4	31193.68	1.423159	**0.310298**	3.837853
Formulation	45800.5	2	22900.25	1.044785	**0.395248**	4.45897
Error	175349	8	21918.62			
Total	345924.2	14				

Tc Anova: Two-Factor Without Replication

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Endothelium	3	2393.617	797.8723	902710.6
Plasma	3	181.763	60.58767	2291.283
Platelets	3	134.621	44.87367	718.44
Packed RBC	3	61.938	20.646	78.03726
Lysed RBC	3	30.89267	10.29756	16.1576
70/30	5	2115.551	423.1102	664403
60/40	5	514.0825	102.8165	30785.51
50/50	5	173.198	34.6396	1339.752

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Biologic	1404727	4	351181.7	2.033793	**0.182308**	3.837853
Formulation	430242.7	2	215121.3	1.245829	**0.338052**	4.45897
Error	1381386	8	172673.3			
Total	3216356	14				

T0 for Lysed RBC - Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
50/50	2	0.159	0.0795	0.000365
60/40	3	0.379	0.126333	0.00146
70/30	2	0.629	0.3145	0.011705

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.063786	2	0.031893	8.510631	**0.036208**	6.944272
Within Groups	0.01499	4	0.003747			
Total	0.078775	6				

T50 for Lysed RBC - Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
50/50	2	0.91	0.455	0.025992
60/40	3	2.399	0.799667	0.005204
70/30	2	4.395	2.1975	0.007565

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.511661	2	1.75583	159.7474	**0.000153**	6.944272
Within Groups	0.043965	4	0.010991			
Total	3.555626	6				

Tc for Lysed RBC - Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
50/50	2	15.114	7.557	2.455328
60/40	3	25.271	8.423667	3.199674

70/30	2	29.824	14.912	0.021632

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	67.64023	2	33.82011	15.24062	**0.013457**	6.944272
Within Groups	8.876309	4	2.219077			
Total	76.51653	6				

CV

Name: Bill Hao Wang

Education:

The University of Western Ontario
Faculty of Engineering – Mechanical and Materials Engineering
2003-2010 BESc.

The University of Western Ontario
Schulich School of Medicine and Dentistry
2003-2010 M.D.

Postgraduate residency program in neurosurgery
Department of Clinical Neuroscience, London Health Sciences Center
The University of Western Ontario, Schulich School of Medicine and Dentistry
2010-2017

Master of Science Candidate
The University of Western Ontario
Department of Medical Biophysics
2013-2016

Related Word Experience:

Summer Research Training Program (SRTP, Schulich School of Medicine and Dentistry)
2007-2009: Tissue mimicking artificial intervertebral discs
NSERC undergraduate summer research student
2004 summer: Nanoindentation and characterization of material properties

Publications:

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