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Title	Studies on developing a water-soluble polymer bearing adjacent cation-aromatic sequence as a novel embolic material
Author(s)	金, 芝萍
Description	配架番号 : 2757
Degree Grantor	北海道大学
Degree Name	博士(医学)
Dissertation Number	甲第15439号
Issue Date	2023-03-23
DOI	https://doi.org/10.14943/doctoral.k15439
Doc URL	https://hdl.handle.net/2115/89977
Type	doctoral thesis
File Information	JIN_Zhiping.pdf



学 位 論 文

Studies on developing a water-soluble polymer bearing adjacent cation-aromatic sequence as a novel embolic material

(新規塞栓物質としてのカチオン-アロマ配列隣接型水溶性高分子の開発に関する研究)

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List of publications

1. Z. Jin, H. Fan, T. Osanai, T. Nonoyama, T. Kurokawa, H. Hyodoh, K. Matoba, A. Takeuchi, J. P. Gong and M. Fujimura. Gluing blood into gel by electrostatic interaction using a water-soluble polymer as an embolic agent. *Proc. Natl. Acad. Sci. U.S.A.*, 2022, 119, e2206685119

List of presentations

1. Zhiping Jin, Hailong Fan, Toshiya Osanai, Takayuki Nonoyama, Takayuki Kurokawa, Hideki Hyodoh, Jian Ping Gong, Miki Fujimura. Fundamental experiemnts on cationic polymer solutions as a novel embolic material. The 37th Annual Meeting of the Japanese Society for Neuroendovascular Therapy, November 25-27, 2021, Fukuoka, Japan.

2. Zhiping Jin, Hailong Fan, Toshiya Osanai, Takayuki Nonoyama, Takayuki Kurokawa, Hideki Hyodoh, Kotaro Matoba, Akiko Takeuchi, Jian Ping Gong, Miki Fujimura. Gluing blood into a gel through electrostatic interactions: A new path of intravascular embolization. 16th congress of world federation of interventional and therapeutic neuroradiology, August 21-25,2022, international conference center, Kyoto, Japan.

Summary

Background and purpose:

Liquid Embolic Agents (LEAs) are widely used for endovascular embolization of cerebrovascular diseases such as cerebral Arteriovenous Malformations and cerebral Aneurysms. LEAs undergo embolization by forming a clot based on their phase transition. Despite the excellent performances of LEAs, all these classical liquid embolic materials are limited by the adhesion of microcatheter to the embolic agent, use of an organic solvent, unintentional catheter retention, and other complications. In mammals, blood clots formed by fibrin polymerization and platelet activation can act as hemostatic agents physiologically or pathologically obstruct vessels. As blood substance such as blood cells and proteins own negatively charged surface, we supposed that cationic polyelectrolytes can aggregate blood substance to achieve embolization. However, the electrostatic interaction between oppositely charged surfaces normally diminishes in saline water owing to the Debye screening effect, which prevents the formation of a blood gel. Therefore, we synthesized a series of water-soluble polymers bearing adjacent cation-aromatic sequences named poly(cation-*adj*- π) (*adj* is short for adjacent and π for aromatic monomer), which can show electrostatic adhesion with negatively charged blood substances in a physiological environment. In this study, we developed and evaluated the newly synthesized material through blood aggregation tests, in vitro injection tests, in vitro extraction tests, biocompatibility tests and in vivo injection test in order to examine its clinical utility.

Materials and methods:

Four different types of poly(cation-*adj*- π) aqueous solutions and poly(cation) aqueous solutions were synthesized in this research. In Vitro: First, 4 different poly(cation-*adj*- π) aqueous solutions as well as different concentrations of same polymer aqueous solutions were added into citrated blood and different contents of blood taken from Sprague-Dawley rats, poly(cation) aqueous solutions were set as control. Physical properties of formed blood gels were tested through Rheological tests. Second, injection force of injecting polymer solutions into clinical needles and catheters were recorded. Third, traction force of blood gel formed by polymer aqueous solutions and citrated blood after injection was also recorded. And Onyx, one of

currently used commercial liquid embolic agents, was set as control. In Vivo: First, polymer aqueous solutions as well as a poorly flowing entangled polymer hydrogel disc were implanted under the dorsal skin for 30 days and 50 days. Peripheral blood was taken for biochemical test at 7th day and 28th day after implantation. Besides, Hematoxylin-Eosin staining and immunohistochemical staining of tissues around the incision were done for histological analyses. Second, quantified dose of poly(cation-*adj*- π) aqueous solutions were injected into rat's femoral artery. Skin color of injected hindlimb were observed and recorded at selected time points. Then, tissue H&E staining of injected hindlimbs were done for intravascular evaluation. Third, poly(cation-*adj*- π) aqueous solutions were mixed with tantalum powders for radiopaque ability. Next, different doses of mixture were injected into rat's femoral artery. Computed tomography (CT) scanning was conducted at selected points after injection. Finally, rat femoral artery injection was repeated by using 10 rat (20 hindlimbs in total) for verifying the reproducibility of this approach.

Results:

In Vitro: First, poly(cation-*adj*- π) aqueous solutions at different concentrations can rapidly interact with blood within 1 min to form a stable blood gel. By contrast, the poly(cation) cannot. Also, poly(cation-*adj*- π) can glue different blood contents together respectively. The Rheological results showed that blood gel formed by poly(cation-*adj*- π) exhibited viscoelastic behavior and a strong frequency dependence of G' , which is larger than the loss modulus G'' over the tested frequency range. Second, poly(cation-*adj*- π) can be smoothly injected through needles and catheters. Third, traction force of the poly(cation-*adj*- π)-based blood gel was much lower than that of Onyx 18 and was similar to that of non-agglomerate samples using normal saline.

In Vivo: first, Hematoxylin-Eosin staining and immunohistochemical staining results of subcutaneous tissues showed no obvious inflammation around the incision in either the polymer or hydrogel groups. Second, the results of in vivo injection test showed that right hindlimb with embolization gradually darkened and became unable to move. Third, embolization conducted by polymer solutions was confirmed by CT scanning after mixing with tantalum powders. The CT results of the one-shot injection (2–3 μ L) indicated that embolization only occurred near

the injection site, while CT results of 0.05 mL injection showed that the mixture had good fluidity and could completely occlude the arteries of the right hindlimb. Besides, successful embolization was also confirmed on the 7th day after injection.

Discussion:

First, according to results of In vitro blood aggregation test, it was found that poly(cation-*adj*- π) aqueous solutions can rapidly interact with blood to form a stable blood gel, while the poly(cation) cannot. This result indicated that cationic monomers both in poly(cation) and poly(cation-*adj*- π) could react with negatively charged substances through electrostatic interactions, but the electrostatic interaction between oppositely charged surfaces normally diminishes in saline water owing to the Debye screening effect, which leading to unstable blood gel. Poly(cation-*adj*- π) formed a stable blood gel with blood due to the aromatic monomers, which enhance the electrostatic interactions of their adjacent cationic residues with the counter surfaces, even in a high ionic-strength medium. Besides, poly(cation-*adj*- π) aqueous solutions could also aggregate blood cell solution (mainly red blood cells) isolated from whole blood into a stable clot. This result further confirmed that poly(cation-*adj*- π) aqueous solutions can glue blood into gel through electrostatic interactions. Besides, Rheological results showed that blood gel formed by poly(cation-*adj*- π) exhibited viscoelastic behavior and a strong frequency dependence of G' , indicated that the blood gel was a solid-like gel. Second, the results of injection force test verified that poly(cation-*adj*- π) aqueous solutions could be injected smoothly. Then, the traction force test showed low traction force of poly(cation-*adj*- π)-based blood gel, which was much lower than that of Onyx 18 and similar to that of normal saline group. These results indicated that the blood gel formed by poly(cation-*adj*- π) showed no obvious adhesion to plastic tubes, thereby considerably reducing the risk of hemorrhage in the traction process. Third, results of subcutaneous implantation test showed poly(cation-*adj*- π) solutions are biologically well adapted. At the same time, result of 50-day hydrogel subcutaneous implantation showed the hydrogel volumes shrank but the shapes did not change, indicating good stability of the polymers in the body. Finally, in vivo embolization capacity of poly(cation-*adj*- π) aqueous solutions was confirmed by rat femoral artery injection model. poly(cation-*adj*- π) aqueous solutions can occlude the vessel through forming blood gels with

gluing blood contents together, which was verified by H&E staining of occluded vessels. Besides, after mixing with the tantalum powders, embolization conducted poly(cation-*adj*- π) solutions could also be confirmed by post-surgery CT scanning, and the clots can be confirmed even on the 7th day after injection.

Conclusion:

In summary, we have demonstrated a conceptual strategy for endovascular embolization. In contrast to existing LEAs, whose embolization is based on the solidification of exotic materials, the water-soluble polymers bearing adjacent cation-aromatic sequences can glue negatively charged blood substances into a gel-like material through electrostatic interactions in normal saline water, whereas common polycations cannot form these interactions. Owing to its easy delivery through a microcatheter with low injection force coupled with biocompatibility and controlled embolization, the poly(cation-*adj*- π) aqueous solution is a promising liquid embolic agent.

List of abbreviations

ACD	anticoagulant citrate dextrose
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATAC	2-(Acryloyloxy) ethyl trimethyl ammonium chloride
BC	blood cells
BUN	blood urea nitrogen
BZA	benzyl acrylate
cAVM	cerebral arteriovenous malformations
CREA	creatinine
CT	computed tomography
DMSO	dimethyl sulfoxide
GLP	good laboratory practice
H&E	hematoxylin and eosin
I.D.	inner diameter
IHC	immunohistochemical
LEAs	liquid embolic agents
nBCA	N-Butyl-2-cyanoacrylate
PPP	platelet-poor plasma
PDEA	2-(2-phenoxyethoxy)ethyl acrylate
PEA	2-phenoxyethyl acrylate
PSEA	2-(phenylsulfanyl)ethyl acrylate
SD	Sprague-dawley

Introductions

Cerebral arteriovenous malformations (cAVM) are an abnormal connection between cerebral arteries and veins. These arteries and veins converge at a vascular nidus in which feeder arteries connect directly to draining veins without any intervening capillaries (Chen et al., 2020; Soulez et al., 2019). Endovascular embolization is a common treatment for cerebrovascular diseases such as cAVM and cerebral Aneurysms. This therapy is based on the use of a variety of embolic agents to occlude one or more abnormal vessels by using an intravascular microcatheter. Depending on the disease type and embolic material, endovascular treatment can be performed either alone or in combination with other methods (Schimmel et al., 2021; Pan et al., 2021). At present, three main types of embolic materials include mechanical occlusion devices, particulates, and liquid embolic agents (LEAs) (Hu et al., 2019). These agents can also be subcategorized into temporary and permanent embolic materials based on their properties (6 Yang et al., 2019). Among them, LEAs are the most common material for cAVM treatment, which are generally triggered by environmental changes between administration (e.g., syringe and catheter) and operating conditions (i.e., blood vessels). LEAs with low viscosities are required to pass through delivery systems and form solid-like materials after exiting. Solidification of these materials can be realized by polymerization, precipitation, or gelation (Ko et al., 2022; Lord et al., 2020; Fan et al., 2020; Oowaki et al., 2000). N-Butyl-2-cyanoacrylate (nBCA) (TruFill®) is a typical polymerization-based liquid embolic agent. nBCA is a water-insoluble liquid monomer that can rapidly polymerize upon contact with an ionic substance (OH⁻) in body fluid in seconds; however, fast polymerization may lead to nontarget embolization and adherence of the catheter to the vessel walls (Vollherbst et al., 2022; Higashino et al., 2021). Precipitation-based liquid embolic agents (e.g., Onyx®) are water-insoluble polymers dissolved in the organic solvent dimethyl sulfoxide (DMSO). When the liquid exits the catheter, DMSO rapidly diffuses into the blood, and the polymer precipitates. The use of organic solvents entails certain safety risks; for example, the rapid injection of DMSO solutions induces vasospasm and can cause neurotoxicity and vascular toxicity (Vollherbst et al., 2022; Mueller et al., 2007). In contrast to the above systems, gelation-based liquid embolic agents are waterborne systems. Such water-soluble polymers usually contain

functional groups that are sensitive to temperature, pH and/or ionic strength. Gelation-based embolic agents require a well-defined gelation time to block lesions while preventing catheter blockage and nontarget embolization (Jones et al., 2016; Poursaid et al., 2016; Kim et al., 2021). Besides, Calcium alginate gel (ALGEL) (Neural Intervention Technologies, Ann Arbor, MI) is a new embolic agent developed primarily for neuroendovascular procedures (Vaidya et al., 2008). Water-soluble alginate can be ionically cross-linked with nontoxic divalent cation solution, such as calcium chloride (Becker et al., 2001). The biocompatibility and embolization properties of calcium alginate gel were tested in rat kidney capsules and rabbit kidneys, respectively (Becker et al., 2001). Moreover, the effectiveness of calcium alginate gel has also been examined in a porcine aneurysm model (Becker et al., 2007). However, separate delivery of alginate and calcium ions is required for the system, e.g., concentric/dual-lumen catheter, to prevent polymerization inside catheter. Thus, meticulous clinical experience is essential for this technique, limiting its use (Vaidya et al., 2008; Soga et al., 2004).

Although present embolic agents showed excellent performances during clinical treatment, certain shortcomings including neurotoxicity and angiotoxicity by using an organic solvent, adhesion of the microcatheter to the embolic agent, incomplete occlusion, and unintentional catheter retention (Hu et al., 2019; Fan et al., 2020; Schmitt et al., 2021) still limit their effectiveness of treatments. Besides, to the best of our knowledge, the clots formed by these liquid embolic agents undergo embolization based on their phase transition.

In mammals, fibrin polymerization and platelet activation lead to a blood clot, which is mainly composed of aggregated blood contents (such as red blood cells). These blood clots can act as hemostatic agents physiologically and pathologically obstruct arteries or veins (Smith et al., 2015). The main substances in human blood are blood cells and proteins, whose surfaces are negatively charged (Walayat et al., 2017). Therefore, the electrostatic interactions between cationic polyelectrolytes and blood substances can be utilized as an alternative route to achieve vascular embolization. However, the electrostatic interaction between oppositely charged surfaces normally diminishes in saline water owing to the Debye screening effect. Debye screening effect is related to the Debye length which was first proposed by the Dutch physicist P. Debye when he was studying the phenomenon of electrolysis. Debye length is a measure of a

charge carrier's net electrostatic effect in a solution and how far its electrostatic effect persists. A buffer or solutions with high concentration of salt, such as normal saline water, has a small Debye length (Israelachvili, 2011). Therefore, electrostatic interaction between oppositely charged surface (cationic polyelectrolytes and blood substances) will diminish in saline water, which prevents the formation of a blood gel.

Therefore, a water-soluble polymer that can glue blood contents together but does not trigger coagulation, as well as overcome the diminishment of electrostatic interaction in saline water, can be developed as a new strategy to achieve embolization while overcoming the above-mentioned shortcomings.

Recently, inspired by the amino acid sequence of protein kinase C, our group synthesized a series of water-soluble polymers bearing adjacent cation–aromatic sequences named poly(cation-*adj*- π) (*adj* is short for adjacent and π for aromatic monomer) (Fan et al., 2019). Aromatics on copolymers were found to enhance the electrostatic interactions of their adjacent cationic residues with the counter surfaces, even in a high ionic-strength medium (Fan et al., 2021). Based on this mechanism, we supposed that this newly synthesized polymers can aggregate blood contents into blood gel to achieve embolization (Fig. 1).

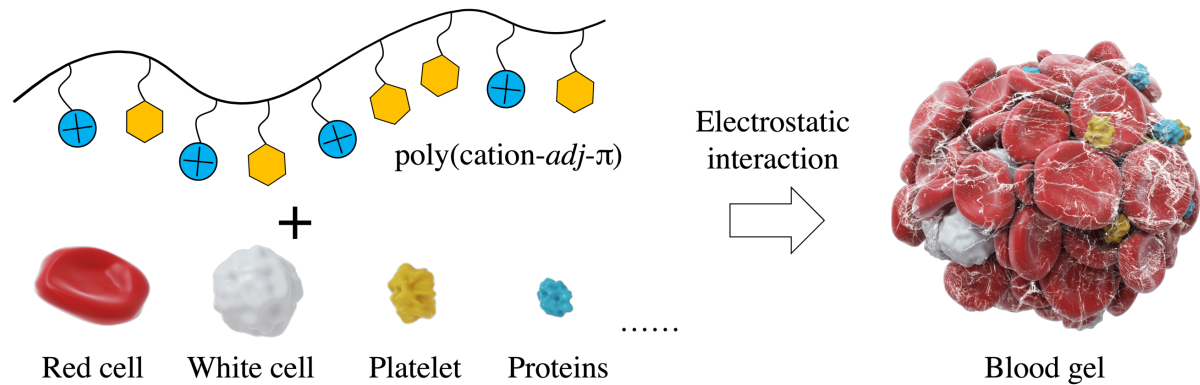


Figure 1 Schematic illustration of blood gel formation strategy

Schematic illustration of *poly(cation-adj- π)* glued the negatively charged blood substances into a blood gel.

In this study, we aim to confirm whether this newly synthesized polymer solutions are capable for endovascular embolization through a series of *in vitro* and *in vivo* experiments.

Methods

1. Preparation of polymer aqueous solutions

1.1. Materials

2-(Acryloyloxy) ethyl trimethyl ammonium chloride (ATAC, 79.4% in water) was provided by Toagosei Co., Ltd., Japan. 2-(Phenylsulfanyl)ethyl acrylate (PSEA), 2-(2-phenoxyethoxy)ethyl acrylate (PDEA), benzyl acrylate (BZA), and 2-phenoxyethyl acrylate (PEA) were provided by Osaka Organic Chemical Co. Ltd., Japan. α -Ketoglutaric acid (UV initiator), DMSO, and tantalum powder were purchased from Wako Pure Chemical Industries, Ltd., Japan. All chemicals were used as purchased without further purification. Millipore deionized water was used in all experiments.

1.2. Synthesis of poly(cation-*adj*- π)

The cationic monomer (0.5 M), aromatic monomer (0.5 M), and UV initiator (2-oxoglutaric acid, 0.25 mM) were first dissolved in DMSO, and then the mixture was polymerized under UV light irradiation (3.9 mW/cm², 11 h) in a glove box. Subsequently, the solution was loaded into a dialysis bag (cut-off Mw: 3500), which was subsequently dipped in a large amount of water to remove DMSO and unreacted chemicals. The water was exchanged every 12 h for one week. Following freeze-drying, pure poly(cation-*adj*- π) was obtained.

1.3. Synthesis of poly(cation)

The cationic monomer ATAC (1.0 M) and UV initiator (2-oxoglutaric acid, 0.25 mM) were first dissolved in water, and then the mixture was polymerized under UV light irradiation (3.9 mW/cm², 11 h) in a glove box. Subsequently, the solution was loaded into a dialysis bag (cut-off Mw: 3500), and the bag was dipped in a large amount of water to remove unreacted chemicals. Water was exchanged every 12 hours for one week. Following freeze-drying, pure poly(cation) was obtained.

1.4. Preparation of polymer aqueous solutions

Pure poly(ATAC-*adj*-PEA), poly(ATAC-*adj*-BZA), poly(ATAC-*adj*-PSEA), poly(ATAC-

adj-PDSA), and poly(cation) were dissolved in Millipore deionized water for obtaining different concentration of polymer aqueous solutions. Besides, poly(ATAC-*adj*-PEA) was also dissolved in normal saline water for obtaining polymer normal saline solutions.

2. In vitro test

All blood samples were extracted from eight-week-old Sprague-Dailey (SD) rats (300–340 g) purchased from CLEA Japan, Inc. (Tokyo, Japan) and stored in anticoagulant citrate dextrose-A (ACD-A) solution purchased from Terumo Corporation Japan (Tokyo, Japan) for anticoagulation.

2.1. Qualitative aggregation test

Qualitative aggregation test was performed based on previous studies (Avery et al., 2016; Gaharwar et al., 2014) to confirm whether polymer solutions can react with whole blood. Briefly, 150 μ L of different types of polymer solutions as well as polymer solutions at different concentrations were added to sequential wells of a 48-well plate, ensuring that the entire bottom surface was coated with polymer. The same volume of citrated blood was then added to each well. At selected time points, normal saline was added to the wells to halt the reaction and aspirated immediately. The well was washed repeatedly until the solution became clear, which indicated the removal of all unaggregated blood components. Citrated blood (150 μ L) with calcium chloride (CaCl_2 , 0.1 M) at a volume ratio of 9:1 was used as a control.

2.2. Quantitative aggregation test

Quantitative aggregation test was performed by weighing the mass of the blood gel to figure out the relationship between amount of used polymer solutions and formed blood gel mass. First, 500 μ L of citrated blood was added to the microcentrifuge tubes. Subsequently, different volumes of poly(cation-*adj*- π) aqueous solutions at different concentrations were slowly added to the blood. After injection, the blood gel was removed immediately and wiped with Kimwipes to remove surface liquid. Subsequently, the weight of the blood gel

was measured by using a microbalance.

2.3. Mechanical properties – Rheological test

Rheological tests were done for confirming the mechanical properties of formed blood gel mass. The tests were performed using an ARES-G2 rheometer (TA Instruments, USA). A rheological angular frequency sweep from 0.01 rad s⁻¹ to 300 rad s⁻¹ was performed with a shear strain of 0.1% in the parallel-plate geometry at 37 °C. Disc-shaped samples with thicknesses of approximately 0.4 mm and diameters of 25 mm were placed on the plates and surrounded by normal saline solution.

2.4. Aggregation of different blood substances

As there are different components in blood, and some of them are closely related to coagulation progress, it is necessary for us to confirm whether different blood components can react with polymer solutions, especially the blood cells.

2.4.1. Preparation of Platelet-poor plasma and blood cells

Platelet-poor plasma (PPP) and blood cells (BC) were obtained by centrifugation of whole blood as previously described (Batista et al., 2018; Gaharwar et al., 2014) to confirm whether different blood substances can react with polymer solutions. Briefly, 9 mL of whole blood was centrifuged (S700FR; Kubota Corporation, Tokyo, Japan) at 160 × g for 20 min to separate plasma from blood cells. Then, the supernatant was pipetted at 4 mm below the dividing line between phases and transferred to a new tube. The remaining BC were transferred to new tubes for further experiments. The second centrifugation for 15 min at 400 × g was performed on the previously obtained supernatants to separate the platelets to the maximum extent. The supernatants of the second centrifugation were used as PPP for further experiments.

2.4.2. Aggregation test of different blood substances

200 μL of polymer solutions were added into PPP and BC obtained by the above method,

respectively. Formed gel was removed immediately and wiped with Kimwipes to remove surface liquid. This process was repeated by three time. Subsequently, the size of all the formed gels were observed.

2.5. In vitro injectability – Injection force test

Injection force test (Jones et al., 2016) was done to confirm the injectability of polymer solutions. The test, using a 1-mL plastic syringe (Nipro Corporation, Osaka, Japan), was performed on a SHIMADZU tester (Autograph AG-X) using the Trapezium X software. The syringe was fixed to the tester to prevent it from moving during testing. The syringe plunger was depressed by a compression plate connected to a 100 N load cell to achieve injection at rates of 1 and 1.5 mL/min. For the needle injection test, poly(cation-*adj*- π) aqueous solutions with different concentrations were added to the syringe (Nipro Corporation, Osaka, Japan) and injected through a 32-gauge needle (HANDAYA Co., Ltd., Tokyo, Japan). For the microcatheter injection test, a polymer solution (40 mg/mL) containing tantalum powders (0.25 mg/mL) was added to the syringe and injected through a 150 cm-long microcatheter (MSV150, Tokai Medical Products Inc., Aichi, Japan), the end of which was placed in a well containing citrated blood. Onyx 18 and normal saline water were used as the references.

2.6. Traction force test

The traction force test was conducted to confirm the adhesiveness between embolic agents and the catheter. The test was performed on a SHIMADZU tester (Autograph AG-X) using the Trapezium X software. A thin polyethylene tube (I.D. 0.28 mm, O.D. 0.61 mm) filled with each sample was inserted into a thick polyethylene tube (I.D. 1.4 mm, O.D. 1.9 mm) containing 0.1 mL citrated blood. Then same amount of polymer aqueous solutions at different concentration as well as Onyx 18 were injected into the blood. After standing for 30 min, the thin polyethylene tube was withdrawn at a rate of 100 mm/min. The force exerted during the pulling process was measured.

3. In vivo test

Eight-week-old SD rats (300–340 g) were purchased from CLEA Japan (Tokyo, Japan) and allowed free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of National University Corporation Hokkaido University (21-0116).

3.1. Biocompatibility and stability test

Biocompatibility and stability test was conducted to confirm the biosafety as well as the stability of this newly synthesized polymer solutions. In this test, subcutaneous implantation was arranged on 18 SD rats (Gaharwar et al., 2014): a 1-cm skin incision was made on the right dorsal side of the rat. A small subcutaneous pocket was then created by blunt preparation. For the experimental group, 50 μ L of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solution (40 mg/mL) and a disc-shaped ($\varnothing$ 5 mm, thickness 1 mm) poly(ATAC-*adj*-PEA) aqueous hydrogel were embedded into the subcutaneous pocket. Then, the incision was anatomically closed, and the rat was allowed to recover from anesthesia. Rats with only a surgical incision were used as sham controls. At a selected time after surgery, blood (serum part) was collected for biochemical tests (SRL, Inc., Tokyo, Japan), and post-perfusion tissues around the incision were collected for histological analyses (Hematoxylin and Eosin (H&E) staining, immunohistochemical (ICH) staining).

In addition, Subcutaneous implantation of another 3 rats were arranged for stability test. Disc-shaped ($\varnothing$ 5 mm, thickness 1 mm) poly(ATAC-*adj*-PEA) aqueous hydrogels were embedded into the subcutaneous pocket as described above. At 50th day after surgery, all the rats were sacrificed, and hydrogel discs were taken out for observation of shape and size.

3.2. Rats' femoral artery embolization

Six SD rats were used to evaluate in vivo embolic effect by rats' femoral artery injection model: A 1-cm incision was made longitudinally on the anterior thigh of the rat's right hindlimb. The femoral artery was exposed through a combination of sharp and blunt dissection under a surgical microscope. Two 7.0 silks (Akiyama Medical MFG. Co., Ltd.,

Tokyo, Japan) were passed underneath the artery to allow for gentle manipulation of the artery by lifting and aligning the vessel for injection. Then, 0.1 mL poly(cation-*adj*- π) aqueous solution (40 mg/mL) was injected distally into the artery using a 32-gauge needle (HANDAYA Co., Ltd., Tokyo, Japan). The incision was then closed anatomically, and the rats were allowed to recover from anesthesia. At 5 and 45 min after surgery, hindlimb skin color was observed and recorded. Finally, all rats were anesthetized and perfused with saline at 4 °C, and the right hindlimb was subjected to histological analysis (H&E staining).

3.3. In vivo post-surgery computed tomography imaging

Post-surgery computed tomography (CT) scanning was done by 6 SD rats for observing new polymer solution mixed with tantalum powders: poly(cation-*adj*- π) aqueous solution (40 mg/mL) containing tantalum powders (0.25 g/mL) was prepared before surgery. Different injection doses were used in the present study. In the first test, ~0.1 mL of the mixture was quickly injected into the right femoral artery of rats in the same procedure as the embolization test. After 45 min, the rats were subjected to CT scanning of the right hindlimbs to confirm embolization. The second test was a one-shot injection (2–3 μ L) of the mixture into the rat's femoral artery using the same procedure mentioned above. After 3 h, the rats were subjected to CT scanning to confirm embolization. All CT examinations were performed as previously described (Hyodoh et al., 2020) using a sixteen slice multidetector row CT (Spuria, Hitachi Corp., Tokyo). The scan parameters were as follows: 120 kV, 215 mA, 0.75 s/rotation, beam pitch 1.3125, collimation 1.25 x 16, slice thickness 1.25 mm. All images were recorded and analyzed using an image workstation (VINCENT, Fujifilm, Tokyo). In addition, another 10 SD rats, 20 hindlimbs in total, were used for reproducibility and stability verification. 10 μ L of the mixture was injected into the femoral artery. On the 7th day after injection, all the rats were taken for CT scanning as described above.

4. Histological analysis

4.1. Hematoxylin and Eosin staining

Hematoxylin and Eosin (H&E) staining was performed for histological analysis. In brief, all

tissues were immersed in 4% paraformaldehyde for over 24 h. Each tissue sample was placed in a processing cassette, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. Next, the paraffin sections (7 μ m) were dewaxed in xylene, rehydrated using decreasing concentrations of ethanol, and washed in deionized water. Then, the sections were subjected to H&E staining. Subsequently, the sections were dehydrated using increasing concentrations of ethanol and xylene and microscopically analyzed.

4.2. Immunohistochemistry

Immunohistochemical (ICH) staining was performed on paraffin-embedded sections (6 μ m thickness) for histological inflammatory analyses (T cells). Briefly, after dewaxing and dehydration, sections were pre-heated using heat-mediated antigen retrieval with sodium citrate buffer (pH 6) for 3 min and then cooled for 20 min. The sections were then incubated with anti-CD3 antibody (ab16669, Abcam, Cambridge, UK) at 1/150 dilution for 60 min at room temperature and detected using an HRP-conjugated compact polymer system. 3,3'-Diaminobenzidine was used as the chromogen. Finally, the sections were counterstained with hematoxylin and then enclosed for observation.

5. Statistical analysis

All data were analyzed using a two-tailed unpaired Student's t-test with unequal Standard Deviation. The mean difference between the control and experimental groups is shown as follows: ns, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

1. Poly(cation-*adj*- π) and poly(cation) aqueous solutions

An adjacent cation–aromatic sequence polymer was synthesized by free-radical copolymerization of equimolar amounts of cationic and aromatic monomers in DMSO at 1.0 M concentration. The polymers were subsequently purified by dialysis and lyophilization, followed by dissolution in water as well as in saline water at different concentrations and ultraviolet sterilization (Fan et al., 2019). Four types of 40 mg/mL poly(cation-*adj*- π) aqueous solution, a kind of 40 mg/mL poly(cation-*adj*- π) normal saline solution were prepared, and 40 mg/mL poly(cation) aqueous solution was prepared for control (Fig.2). Besides, different concentrations (30 mg/mL, 40 mg/mL, 50 mg/mL) of poly(ATAC-*adj*-PEA) aqueous solution were also prepared for experiment.

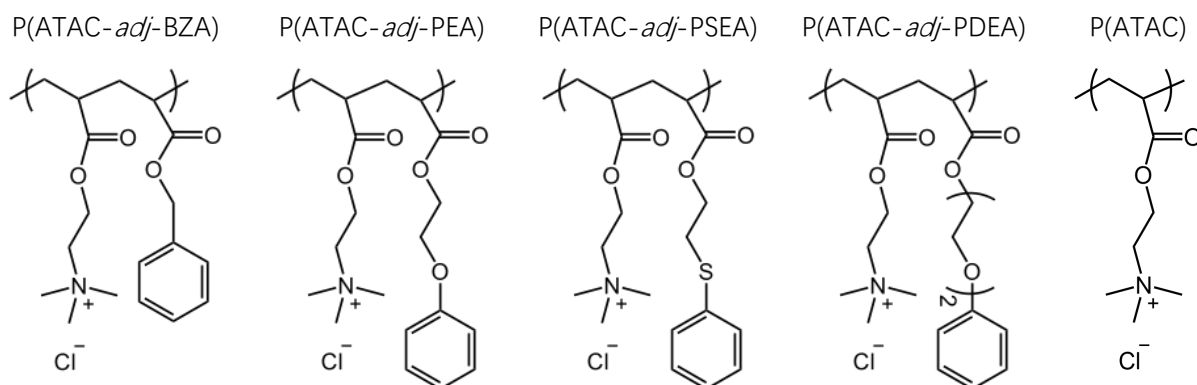


Figure 2 Chemical structures of poly(cation-*adj*- π) and poly(cation)

Chemical structures of 4 types of poly(cation-*adj*- π) and chemical structures of poly(cation).

2. In vitro qualitative aggregation test

Different types of poly(cation-*adj*- π) aqueous solutions, different concentrations of poly(ATAC-*adj*-PEA) aqueous solution as well as poly(ATAC-*adj*-PEA) saline solution were mixed with citrated blood collected from SD rats to confirm whether poly(cation-*adj*- π) can interact with blood. Figure 3 shows that poly(cation-*adj*- π) at different concentrations, as well as different types of poly(cation-*adj*- π) at same concentration can rapidly interact with blood within 1 minute to form a stable blood gel. Besides, poly(ATAC-*adj*-PEA) saline

solution can also react with blood but cannot form a stable blood gel after washed by saline water like polymer aqueous solutions did. By contrast, the cationic homopolymer P(ATAC) cannot form a stable blood gel after washing with normal saline. As a control, we added CaCl_2 to activate the coagulation. The results showed that a stable coagulum could not be formed in a short time.

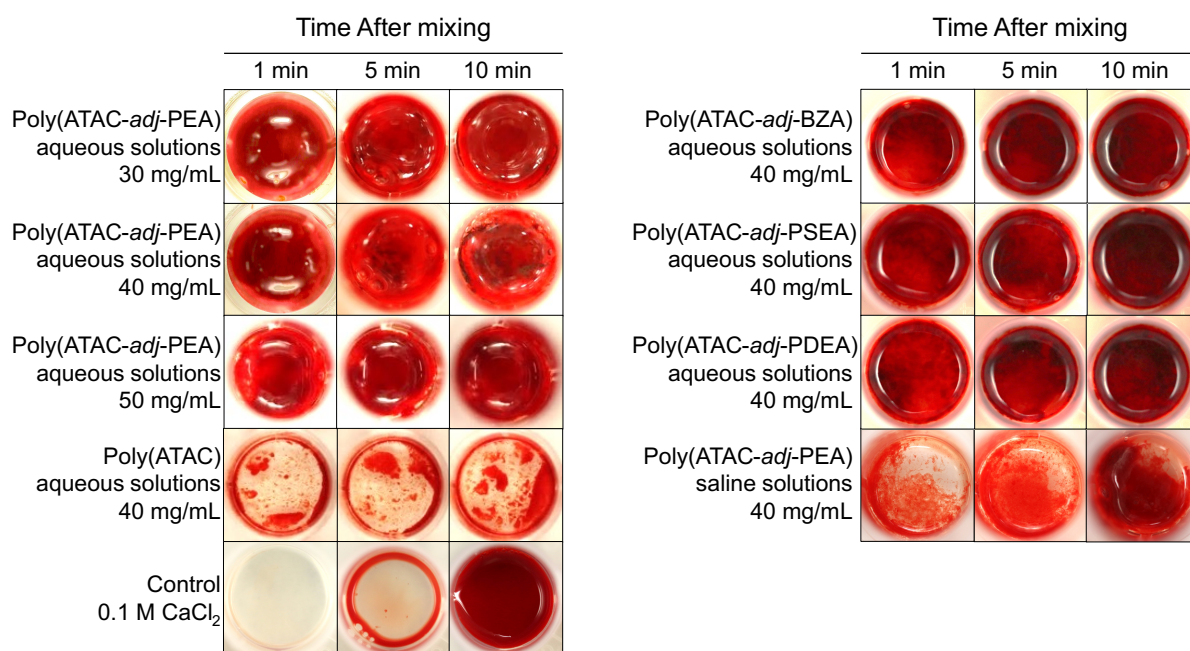


Figure 3 In vitro qualitative aggregation test

Blood formation as a function of time and polymers. All types of poly(cation-*adj*- π) aqueous solutions can react with blood within one minute to form a stable blood gel, while poly(cation) aqueous solutions cannot. Poly(ATAC-*adj*-PEA) normal saline solutions can also react with blood but cannot form a stable blood gel after washed by saline water.

3. In vitro quantitative aggregation test of poly(cation-*adj*- π) aqueous solutions

We compared the mass of the blood gel formed by adding 150 μL of different poly(cation-*adj*- π) aqueous solutions into citrated blood (0.5 mL). No obvious difference in blood gel mass was observed between polymers (Fig. 4).

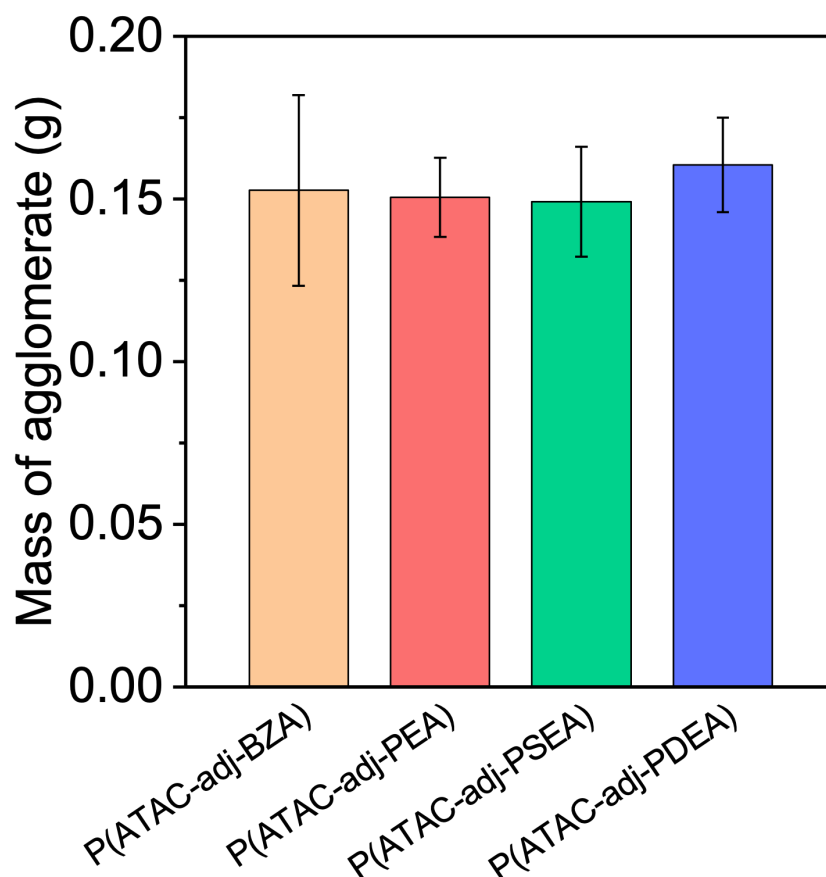


Figure 4 In vitro quantitative aggregation test of poly(cation-*adj*- π) aqueous solutions
Weight of blood gel formed by mixing 0.5mL citrated blood with 150 μ L of 40 mg/mL poly(cation-*adj*- π) solutions.

According to the results of qualitative aggregation and quantitative aggregation, no significant difference was observed between each poly(cation-*adj*- π) aqueous solutions in the formation of the blood gel. Besides, no difference was observed between each concentration of poly(ATAC-*adj*-PEA) aqueous solutions. Therefore, 40 mg/mL poly(ATAC-*adj*-PEA) was selected as a representative for the subsequent in vitro and in vivo experiments to check the potential application of poly(cation-*adj*- π) aqueous solutions as a liquid embolic agent.

3. In vitro quantitative aggregation test for different concertation of poly(ATAC-*adj*-PEA)

To investigate the relationship between the amount of polymer and blood gel mass, different amounts of poly(ATAC-*adj*-PEA) aqueous solutions at different concentrations were added to citrated blood (0.5 mL) and, and the formed blood gel was weighed (Fig. 5). The results

showed that, with an increase in the volume of the polymer aqueous solution, the mass of the blood gel increased and then plateaued with a similar mass. The plateau start point shifted to higher values with a decrease in polymer concentration, but the polymer mass at each point was similar.

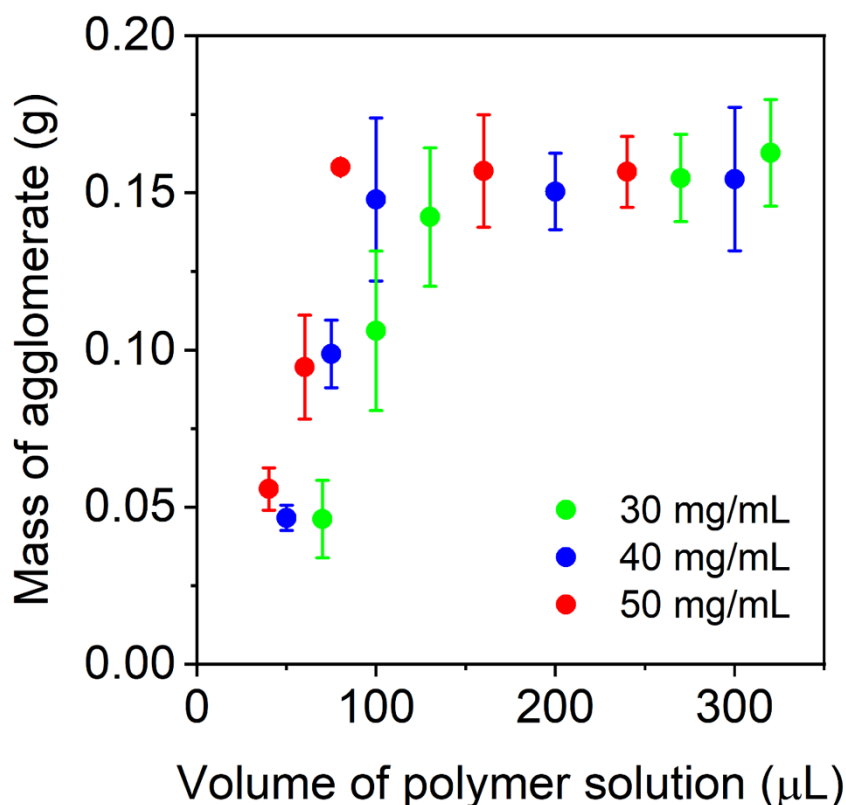


Figure 5 Quantitative aggregation test for different concentration of poly(ATAC-*adj*-PEA) Weight of blood gel formed by mixing 0.5mL citrated blood with different amount of poly(ATAC-*adj*-PEA) aqueous solutions.

4. Mechanical properties of the blood gel – rheological test

The mechanical properties of the blood gel were measured using a rheometer. Rheological results showed that the blood blot prepared by adding CaCl_2 to citrated blood had a frequency-independent storage modulus G' . By contrast, the blood gel formed by poly(ATAC-*adj*-PEA) aqueous solutions exhibited viscoelastic behavior and a strong frequency dependence of G' , which is larger than the loss modulus G'' over the tested

frequency range (Fig. 6A-6C). After prolonged soaking in normal saline water, the blood gel became contracted and squeezed out the fluid contents, leading to an increase in modulus (G' and G'') compared with those of the as-prepared state.

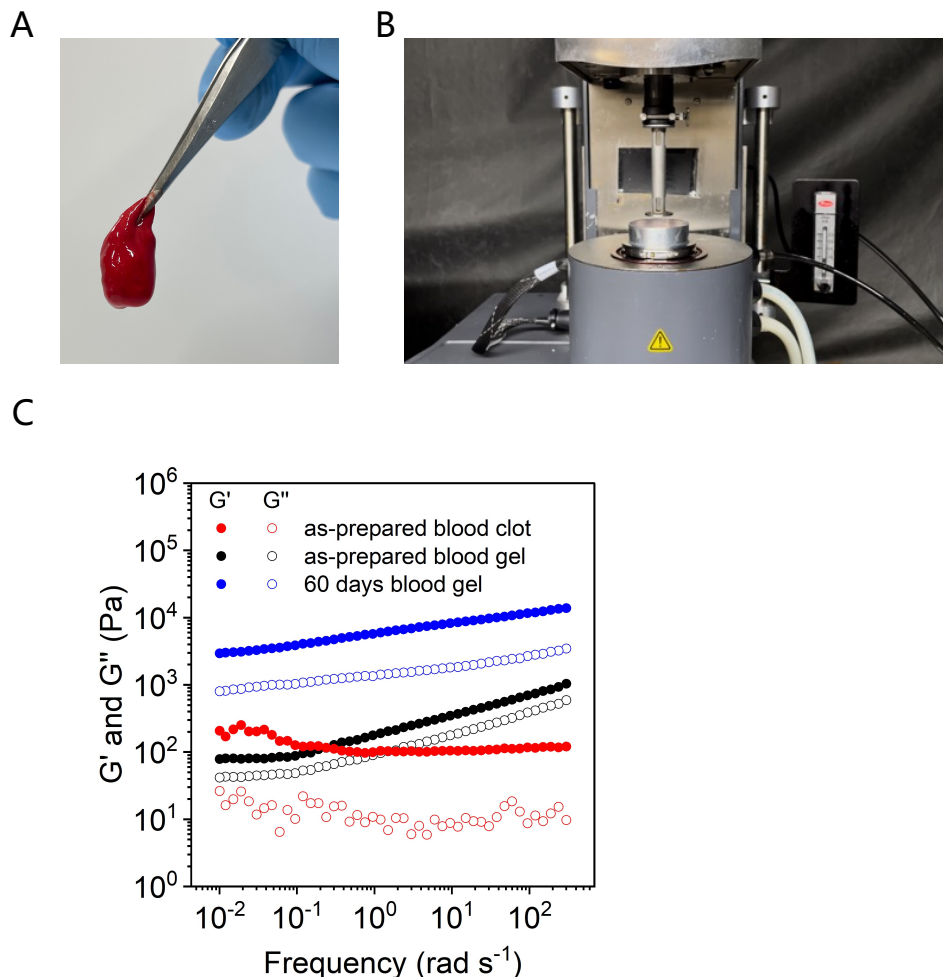


Figure 6 Rheological results of blood gels and blood clots

(A) Blood gel formed by poly(ATAC-*adj*-PEA) aqueous solutions. (2) Rheometer for mechanical properties measurement. (C) Rheological behaviors of blood gel at the as-prepared state and after soaking in normal saline for 60 days. Blood clots formed by mixing 1.0 mL citrated blood with 1.0 mL of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solutions.

5. Aggregation of different blood substance

Considering the multiple substances in the blood, citrated rat blood was centrifuged to obtain PPP and BC (mainly red blood cells) (Shahidi et al., 2018). Then, the obtained contents were

mixed with poly(ATAC-*adj*-PEA) aqueous solutions, and both PPP and BC formed a stable agglomerate with poly(ATAC-*adj*-PEA) aqueous solutions (Fig.7A). Histological photomicrographs (H&E staining) confirmed the absence of an obvious network structure in eosin-stained red blood cell agglomerates (Fig. 7B-7D).

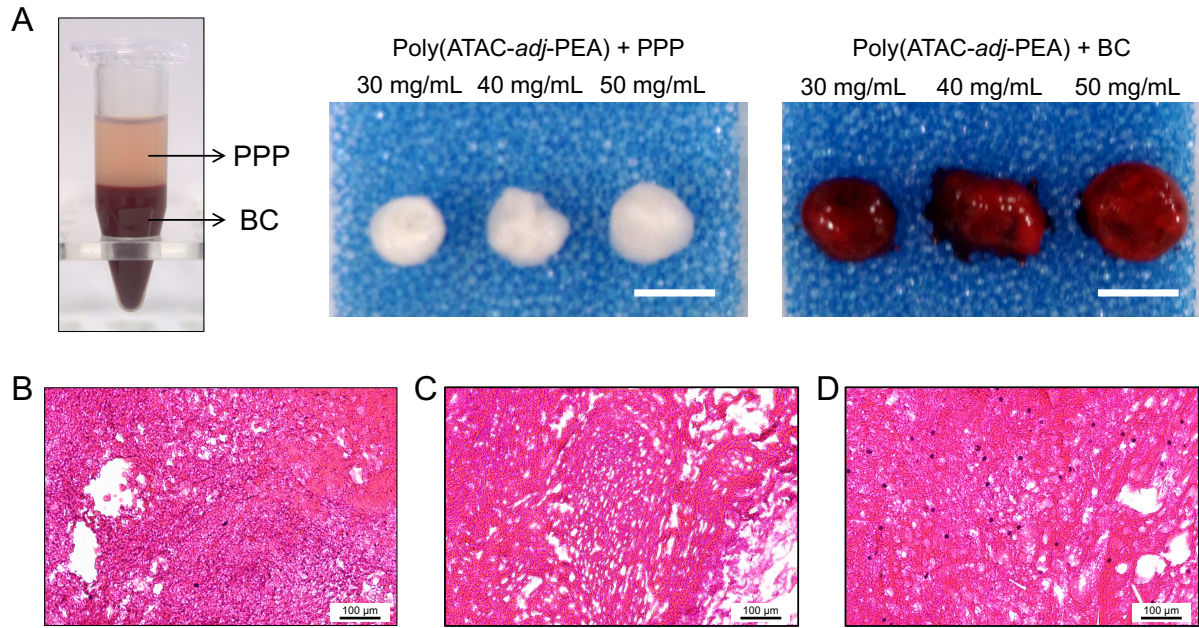


Figure 7 Aggregation of different blood substance

(A) Digital picture of blood gel by mixing 200 μ L of 40mg/mL poly(ATAC-*adj*-PEA) aqueous solutions with 200 μ L PPP and BC, respectively. Scale bars, 0.5cm. (B) H&E staining of the agglomerate formed by blood cells (mainly red blood cells) and poly(ATAC-*adj*-PEA) aqueous solutions. A large area of eosin-stained red blood cells without a network structure has been observed. (C) H&E staining of agglomerate formed by platelet poor plasma and poly(ATAC-*adj*-PEA) aqueous solutions. Alveolar-like structures have been observed. (D) H&E staining of agglomerate formed by whole blood and poly(ATAC-*adj*-PEA) aqueous solutions. Both eosin-stained cells and alveolar-like structure were observed, and hematoxylin-stained cell nuclei were visible (purplish blue).

6. In vitro injectability – injection force test

To confirm injectability, we replicated the injection process by injecting poly(ATAC-*adj*-PEA) aqueous solutions at different concentration through clinical needles and catheters.

The injection force was recorded using a mechanical tester. First, we measured the injection force of the polymer aqueous solutions using 32-gauge clinical needles (inner diameter, I.D. 0.26 mm), as shown in Figure 8A. The results showed that poly(ATAC-*adj*-PEA) aqueous solutions can be smoothly injected through needles. Polymer concentration and injection rate significantly affected the injection force, which first increased and then plateaued in all tests (Fig. 8B-8C). A high polymer concentration leads to a high solution viscosity, resulting in a large injection force.

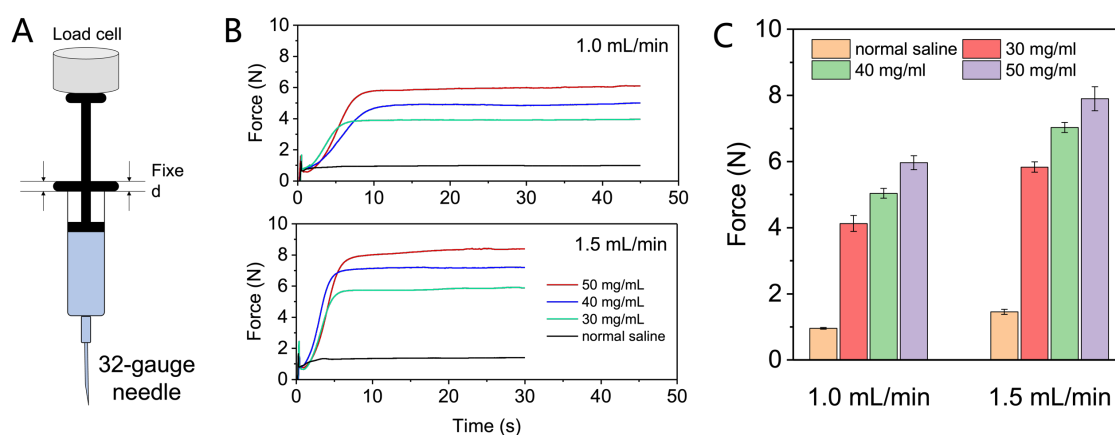


Figure 8 In vitro injection force test (clinical needles)

(A) Schematic illustration of injection force test by using a 1 mL syringe with 32-gauge needle. (B) Injection force curves of poly(ATAC-*adj*-PEA) aqueous solutions and normal saline solution through a 32-gauge needle under different injection rates. (C) Injection force values at the plateau region of poly(ATAC-*adj*-PEA) aqueous solutions and normal saline solution through a 32-gauge needle. The error bars indicate SD (N = 3).

To further verify the injectability of the polymer aqueous solutions in clinical treatment, poly(ATAC-*adj*-PEA) aqueous solutions was mixed with a contrast agent (tantalum powders, 0.25 g/mL, Fig. 9A-9B), and the injection force of mixtures through clinical microcatheters (I.D. 0.43 mm, length 150 cm), whose port was immersed in the citrated blood bath was measured (Fig. 9C). To imitate the discontinuous injection during real operation, the program was set to continue the injection for 7 s and then pause for 3 s, and then run for a total of

four times. For the poly(ATAC-*adj*-PEA) aqueous solutions, the force increased to ~10 N during injection and decreased during the pause in each cycle. For comparison, the same process was used to test the clinical embolic agent Onyx 18. The result showed that injection force of Onyx 18 continually increased after each pause (Fig. 9D).

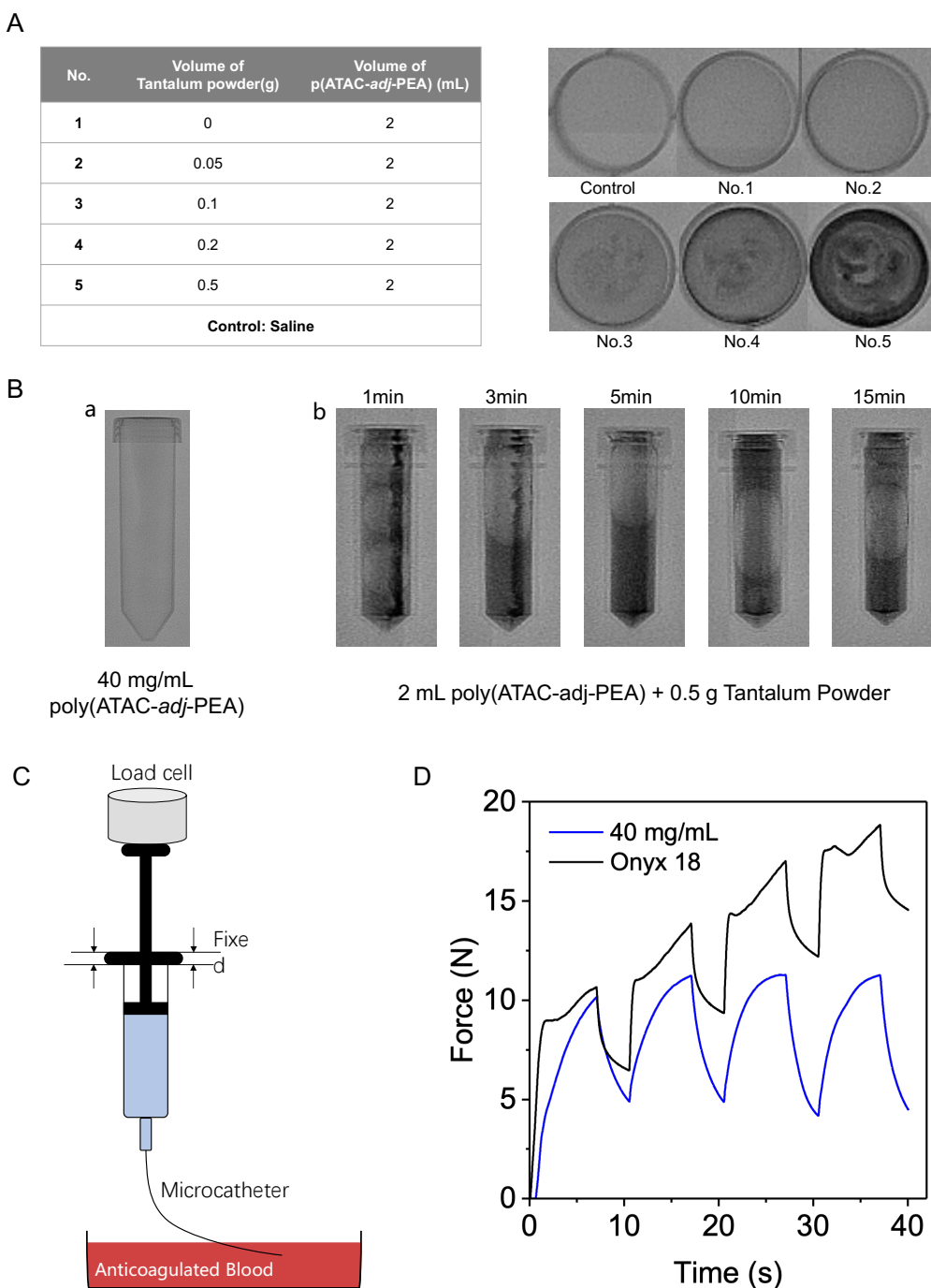


Figure 9 In vitro injection force test (clinical microcatheter)

(A) X-ray images of poly(ATAC-*adj*-PEA) aqueous solutions/tantalum powder mixtures at

different ratios. The concentration of poly(ATAC-*adj*-PEA) aqueous solutions is 40 mg/mL. Normal saline was selected as control. The mixture was stirred by hand for 10 s in petri dish. (B) X-ray images of poly(ATAC-*adj*-PEA) aqueous solutions /tantalum powder mixtures prepared for different mixing times. The mixture was shaken by a shaker (BMS-SMN03, Japan, 2700 rpm) for 1-15 min. The concentration of poly(ATAC-*adj*-PEA) aqueous solutions was 40 mg/mL. Results showed that the mixture became homogeneous after 5 min of shaking. (C) Schematic illustration of injection force test by using a 1 mL syringe with microcatheter. (D) Injection force curves of poly(ATAC-*adj*-PEA) aqueous solutions and Onyx 18 through a 150 cm long microcatheter with 1.0 mL/min injection rate. The program was set to continue the injection for 7 s and then stop for 3 s and repeated for four times.

7. In vitro traction force test

An in vitro traction test was conducted to confirm the adhesiveness between embolic agents and the catheter. As shown in Figure 10A, a thin polyethylene tube (I.D. 0.28 mm) was inserted into a thick polyethylene tube (I.D. 1.4 mm) filled with 0.5 mL citrated rat blood, and the same volume of poly(ATAC-*adj*-PEA) aqueous solutions was injected into the middle part of the blood through a thin polyethylene tube. After standing for 30 min, the thin polyethylene tube was withdrawn, and the force used during the pulling process was simultaneously recorded. Figures 10B and 10C show that the traction force of the poly(ATAC-*adj*-PEA)-based blood gel was much lower than that of Onyx 18 and similar to that of non-agglomerate samples using normal saline.

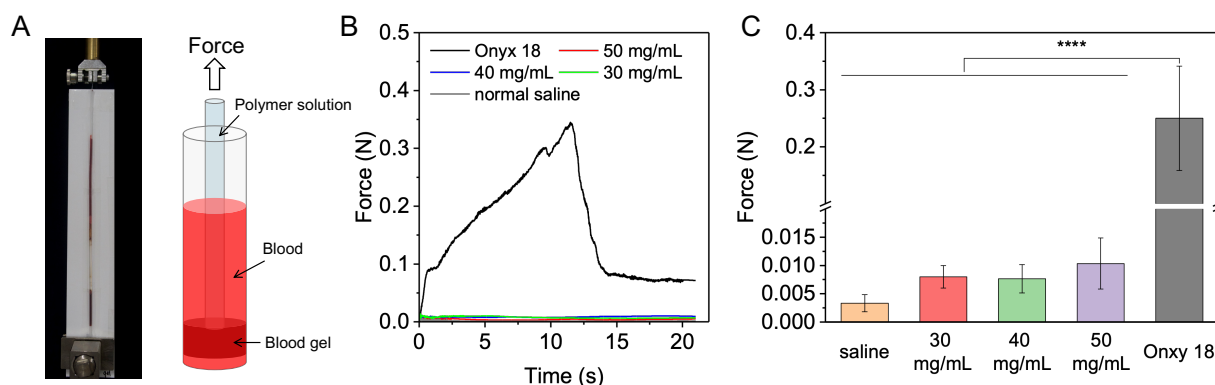


Figure 10 In vitro traction force test

(A) Digital photo and scheme illustration of traction force measurement. (B) Traction force curves of polyethylene tube pulling out of the blood after the blood gel was formed by poly(ATAC-*adj*-PEA) aqueous solutions and Onyx 18. (C) The maximum force values during traction. The error bars indicate SD (n = 3 in each group), ***p < 0.001.

8. Biocompatibility and stability of poly(cation-*adj*- π).

To confirm the biocompatibility of poly(cation-*adj*- π) and enable easy observation, a polymer solution and a poorly flowing entangled polymer hydrogel were used to conduct the experiment. In the experimental group, 50 μ L of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solution and a disc-shaped ($\varnothing$ 5 mm, thickness 1 mm) poly(ATAC-*adj*-PEA) aqueous hydrogel were implanted under the dorsal skin for 7 and 28 days, respectively, and rats with skin incision only were used as sham controls. In addition, we implanted the poly(ATAC-*adj*-PEA) aqueous hydrogel under the dorsal skin for 50 days to evaluate its stability in the body. Clinical serum biochemical tests and histological analyses of skin tissues were performed for comparison. In this study, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used to assess liver function after polymer or hydrogel plantation, whereas creatinine (CREA) and blood urea nitrogen (BUN) were used to assess renal function. Then, H&E staining and immunostaining (CD3, Abcam, USA) were simultaneously conducted to confirm whether severe inflammatory response occurred after implantation.

After surgery, all rats were in good health, and no swelling or redness was observed on the skin around the incision (Fig. 11A). According to figure 11B, Shrinkage of hydrogel discs

without any shape change was observed and no significant abnormalities were observed in polymer solution groups. On the 50th day after surgery, the hydrogels were taken out. The results further confirmed that the hydrogel volumes shrank but the shapes did not change (Fig. 11C).

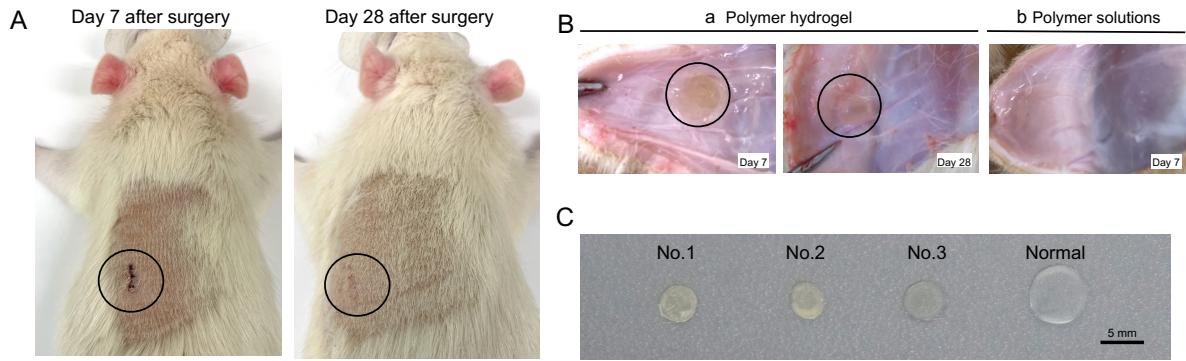
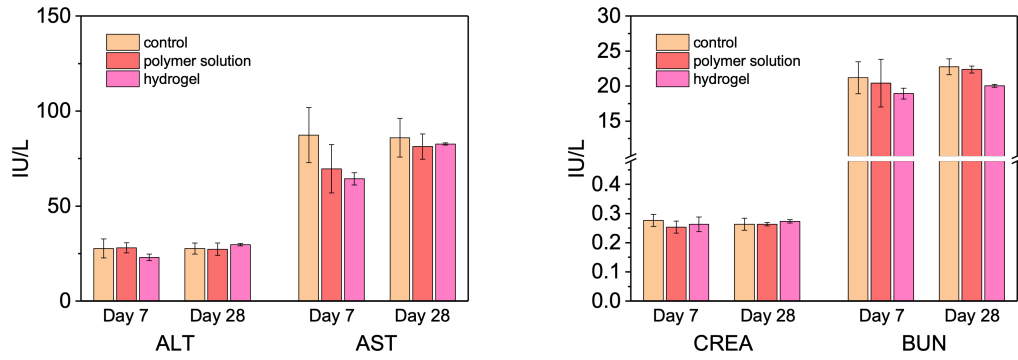


Figure 11 Biocompatibility and stability of poly(cation-*adj*- π).

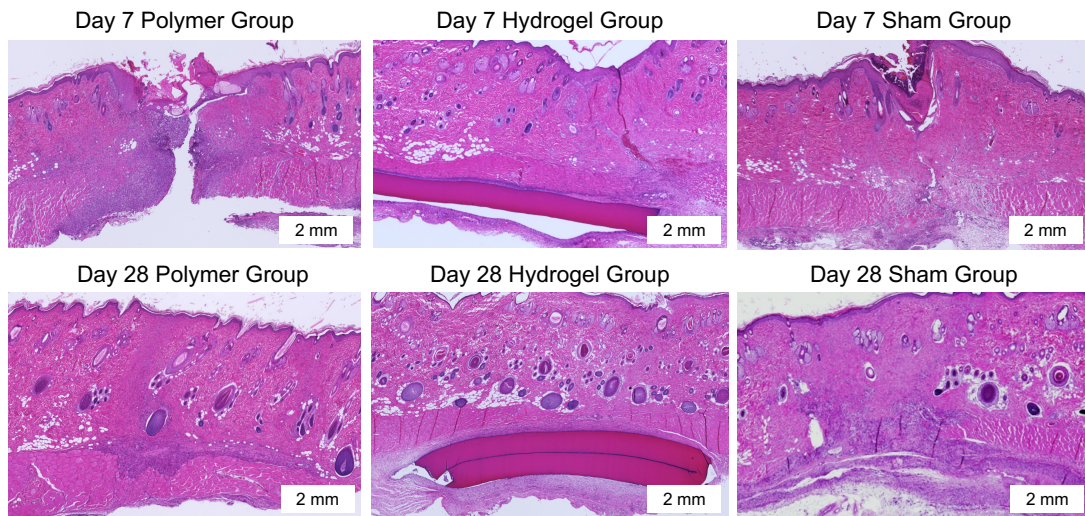
(A) Digital photos of subcutaneous implantation (dorsal surgery incision) in rats after 7- days and 28-days post-surgery. Dorsal surgery incision without swelling and redness on 7 and 28 days after implantation was observed. (B) Subcutaneous conditions after poly(ATAC-*adj*-PEA) aqueous hydrogel disc and aqueous solution implantation. a) Shrinkage of hydrogel discs without any shape change was observed. b) No significant abnormalities were observed. (C) Digital photos of poly(ATAC-*adj*-PEA) aqueous hydrogels before and after 50 days implantation.

As shown in Figures 12A, compared to those of rats in the sham group, four parameters did not increase in tested rats within four weeks after hydrogel and polymer aqueous solution implantation. Besides, H&E staining and ICH staining results also showed no obvious inflammation around the incision in either the polymer aqueous solution or hydrogel groups (Fig. 12B-12C).

A



B



C

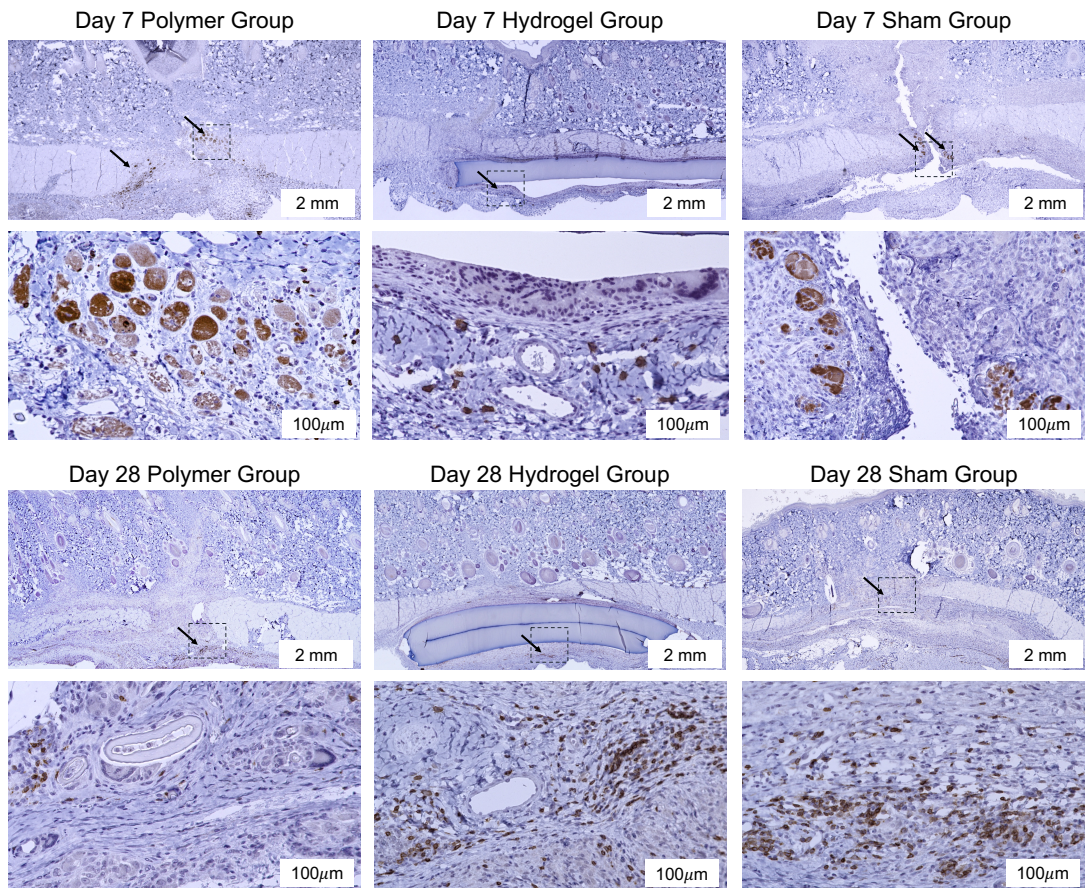


Figure 12 Biochemical and histological examination after subcutaneous implantation.

(A) Serum biochemical tests of liver function of rats at 7 and 28 days after implantation. Data are shown as mean $\pm$ standard deviation, ns, $p > 0.05$. (B) Histological photomicrographs (H&E staining) of the subcutaneous inflammatory response at 7 and 28 days after implantation. (C) Histological photomicrographs (ICH staining) for CD3 positive staining of subcutaneous tissues around the incision (as indicated by black arrows).

9. In vivo embolization in rat femoral artery

To assess the possibility of in vivo non-target embolization of the poly(cation-*adj*- π) aqueous solution, 0.1 mL of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solution was injected into the right femoral artery of rats to form an embolism (Fig. 13A). After surgery, the right hindlimb with embolization gradually darkened and became unable to move (Fig. 13B). Furthermore, H&E staining clearly showed occlusion clots left in vessels. Red blood cells and nuclei of white cells were observed in this occlusion clots (Fig. 13C).

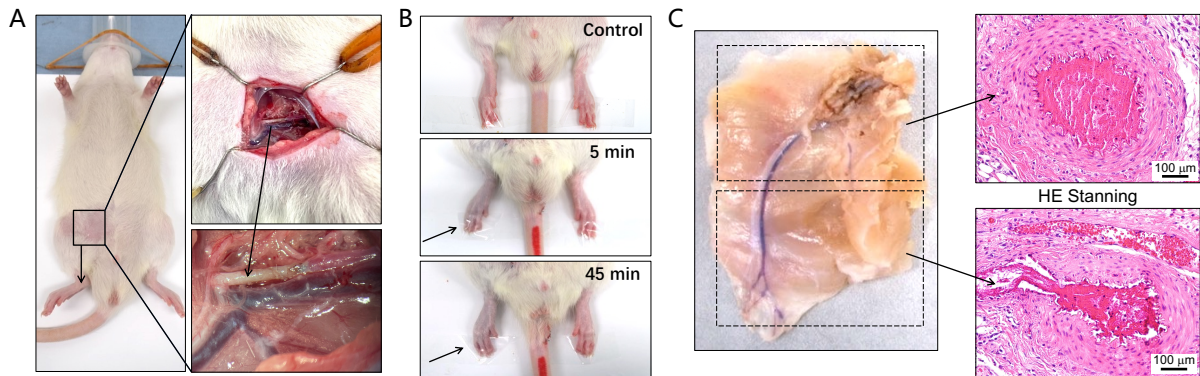


Figure 13 In vivo embolization test

(A) Digital photos of surgery area and rat's femoral artery after injection of poly(ATAC-*adj*-PEA) aqueous solution through a 32-gauge needle. The left arrow indicated the distal injection, and the right arrow indicated the injection location. (B) Digital photos of skin color change on rat's right hindlimb after surgery. (C) Histological photomicrographs of the right hindlimb with embolization.

10. In vivo post radiopacity of poly(cation-*adj*- π)

To further confirm the post radiopacity of poly(cation-*adj*- π), 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solution containing tantalum powders (0.25 g/mL) was prepared for the experiment (Fig. 14A). Then, the mixture was injected in a single shot (2–3 μ L) in the right femoral artery of rats, and CT scanning was conducted at 3 hours after injection. The CT results of the one-shot injection (Fig. 14B and 14D) indicated that embolization only occurred near the injection site instead of flowing far forward with the blood. Then, 0.05 mL of the mixture was further injected into the rats' right femoral artery, which was subsequently CT scanned at 45 min after injection. The CT results (Fig. 14C and 14E) showed that the mixture had good fluidity and could completely occlude the arteries of the right hindlimb.

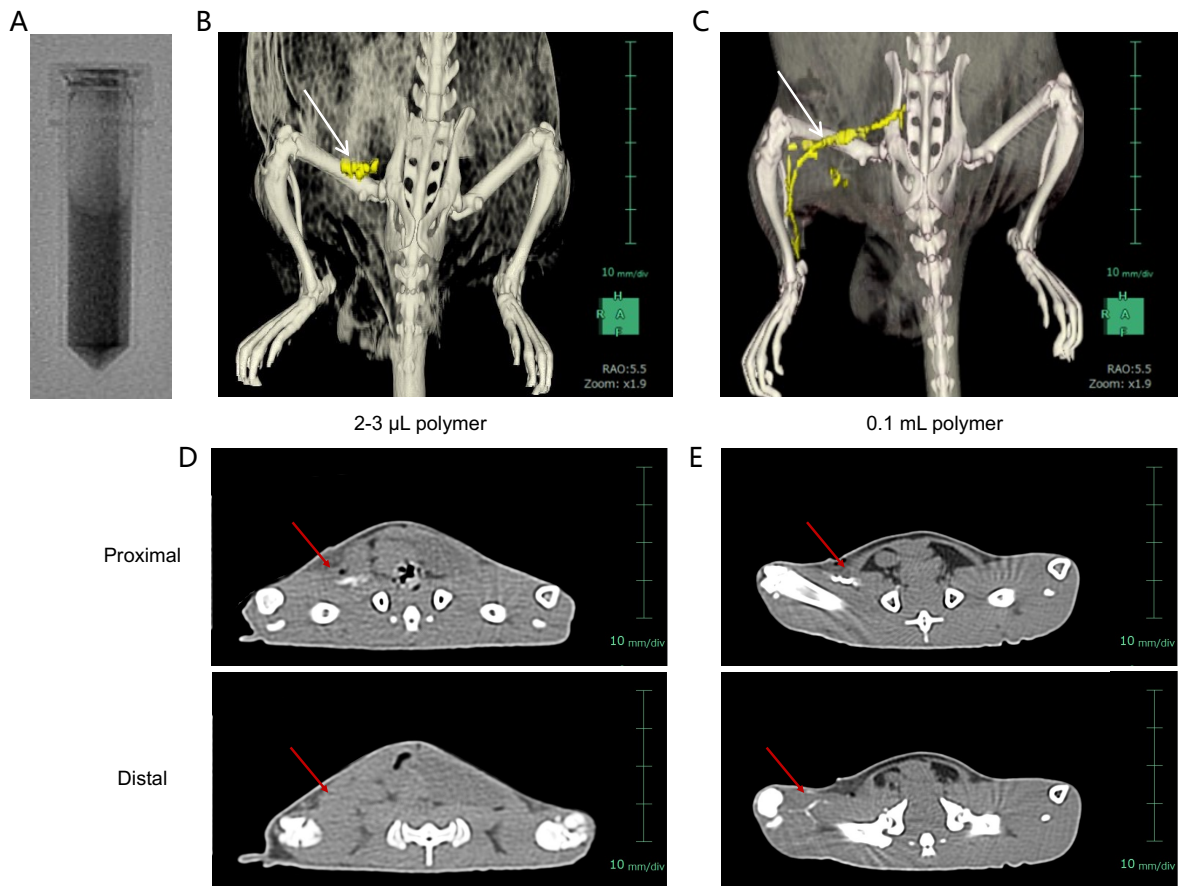


Figure 14 In vivo post-surgery computer tomography (CT) imaging

(A) X-ray image of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solution containing tantalum powders (0.25 g/mL) after 5 min of vibration. (B) CT image of rat after injection of 2–3 μ L poly(ATAC-*adj*-PEA) aqueous solution. (C) CT image of rats after injection of 0.05 mL

poly(cation-*adj*- π) aqueous solution. Arrows indicate the radiopaque polymer, which is artificially colored yellow. (D) Lower limbs of rats injected with 2–3 μ L of 40 mg/mL poly(ATAC-*adj*-PEA). Hyperdense spots were visible near proximal side (red arrow), while no hyperdense spots were found in the distal side. (E) Lower limbs of rats injected with 0.05 mL of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solutions. Hyperdense spots were found in both proximal and distal sides (red arrows).

To verify the reproducibility of this approach, we have repeated the rat femoral artery injection experiment by using 10 rats (20 hindlimbs in total), among which, 10 hindlimbs were injected with P(ATAC-*adj*-PEA) aqueous solutions (40 mg/mL, $\sim$ 10 μ L), another 10 hindlimbs were injected with normal saline water ($\sim$ 10 μ L) as a control. After injecting the polymer solutions, the skin of hindlimbs became dark and unable to move. On the 7th day after injection, all rats were sacrificed and underwent CT scanning of hindlimbs. The CT 3D reconstruction images confirmed that all the femoral arteries in experimental group were occluded by the blood gels. In contrast, the hindlimbs injected with saline were healthy (Fig. 15).

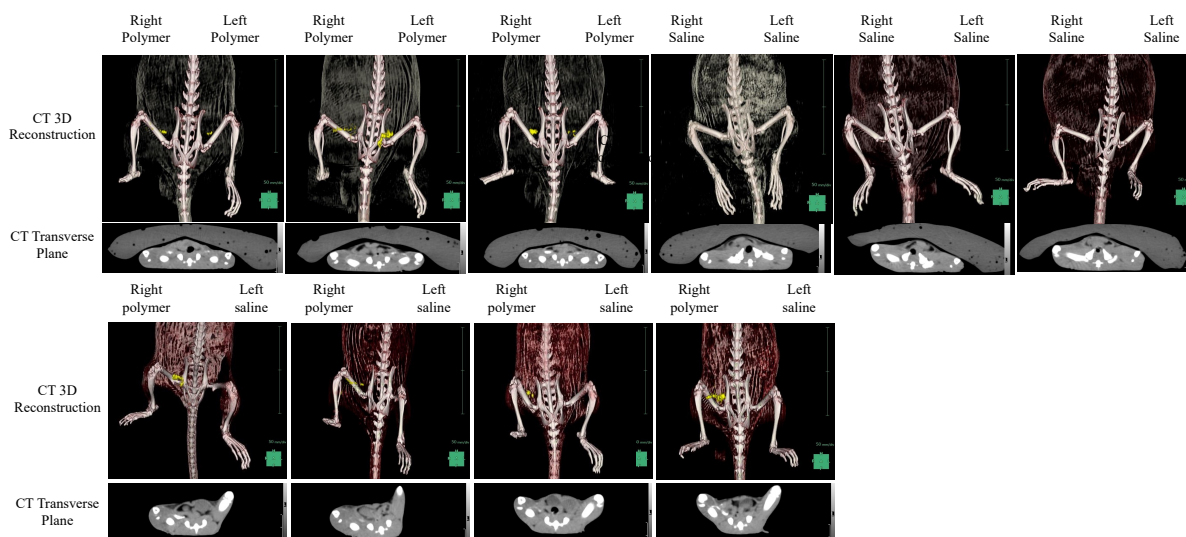


Figure 15 In vivo post-surgery computer tomography (CT) imaging (reproducibility).

Digital photos of rat's hindlimb and CT image of rat on the 7th day after injection of $\sim$ 10L poly(ATAC-*adj*-PEA) (40 mg/mL) aqueous solution or normal saline water.

Discussion

In this research, we developed a water-soluble polycation that can glue negatively charged blood contents together through electrostatic interactions but not trigger coagulation. Further, blood gel formed by this water-soluble polycation can embolize the vessels successfully.

1. The aggregation ability of poly(cation-*adj*- π)

In mammals, fibrin polymerization and platelet activation lead to a blood clot, which is mainly composed of aggregated blood contents (such as red blood cells). These blood clots can act as hemostatic agents physiologically and pathologically obstruct arteries or veins. As the main substances in human blood are blood cells and proteins, whose surfaces are negatively charged, we supposed that electrostatic interactions between cationic polyelectrolytes and blood substances can be utilized to achieve vascular embolization. However, the electrostatic interaction between oppositely charged surfaces normally diminishes in saline water owing to the Debye screening effect, which prevents the formation of a blood gel. To solve this problem, our group synthesized a series of polymers bearing adjacent cation–aromatic sequences named poly(cation-*adj*- π) (*adj* is short for adjacent and π for aromatic monomer) (Fan et al., 2019). Aromatics on copolymers were found to enhance the electrostatic interactions of their adjacent cationic residues with the counter surfaces, even in a high ionic-strength medium (Fan et al., 2021). Thus, we supposed that this newly synthesized polymers can aggregate blood content into blood gel for embolization.

Pure poly(cation-*adj*- π) can be either dissolved in water or normal saline water. Therefore, we prepared poly(cation-*adj*- π) aqueous solutions, poly(cation-*adj*- π) normal saline solutions, and poly(cation) aqueous solutions. First, we confirmed the aggregability between blood contents and these polymers. The result showed both poly(cation-*adj*- π) aqueous solutions and poly(cation-*adj*- π) normal saline solution can react with blood and form a blood gel. However, poly(cation) aqueous solutions, without the existence of aromatics, cannot form strong electrostatic interactions in saline water owing to the Debye screening effect. Further, we compared the aggregability poly(cation-*adj*- π) aqueous solutions and poly(cation-*adj*- π) normal

saline solution. Although both of them can form a blood gel after mixing with blood, the stability of the blood gel was different. In aqueous solution group, polymer can react with blood and form a stable blood gel in a short time (within one minute). However, in normal saline group, formed blood gel was not stable after washed by normal saline. Theoretically, normal saline is a kind of better solvent than aqueous solutions. However, polymer normal saline solutions cannot form stable blood gels with blood. This is due to debye screening effect, by which the repulsive force between cations was shielded. Then the attraction between cation and benzene makes the chain become entangled, which affect the electrostatic interaction between polymer and negatively charged surface. Besides, these unstable blood gel may affect embolization and even cause complications in when used in vivo. Therefore, we only chose poly(cation-*adj*- π) aqueous solutions for the following experiment in this research.

Because the blood gel was formed by the blood contents and poly(cation-*adj*- π) aqueous solutions, the relationship between the amount of polymer and blood gel mass needs to be confirmed. the result quantitative aggregation test indicated the existence of a saturation mass of formed blood gel within a certain volume of blood that is not related to the amount of added polymer. This finding confirms that the blood gel is mainly composed of substances in the blood, while the poly(cation-*adj*- π) works as a glue to bind them together. Moreover, the proper injection volume can be calculated after preoperative assessment, which not only prevents the overuse of the polymer but also reduces the cost of treatment.

As no significant difference was observed between each polymer in the formation of the blood gel; poly(ATAC-*adj*-PEA), a well-studied type, was selected as a representative for the following in vitro and in vivo experiments in this research.

We measured the mechanical properties of the blood gel by using a rheometer. Rheological results indicated that the blood blot prepared by adding CaCl_2 to citrated blood possessed an elastic polymer network, which was consistent with the notion that clots are composed of a three-dimensional network of fibrin fibers (Kaibara, 1996). By contrast, the results of blood gel formed by poly(ATAC-*adj*-PEA) aqueous solutions indicated that the blood gel was a solid-like gel. After prolonged soaking in normal saline water, the blood gel became contracted and squeezed out the fluid contents. These results implied the stability of the blood gel and

supported further investigation into the potential of poly (cation-*adj*- π) aqueous solutions as a liquid embolic agent.

Normally, blood clot formation (coagulation) is related to plasma proteins (fibrin polymerization) and platelet activation, a process that entails a complicated cascade of enzyme activation events. Usually, fibrinogen is enzymatically converted to fibrin by thrombin, forming a fibrin-based blood clot. Thrombin is produced by the enzymatic cleavage of prothrombin through activated factor X and its cofactor (factor V) in conjunction with calcium. Therefore, we supposed that if we can verify the aggregation between polymer solutions and different blood contents respectively, then we can consider that the aggregation is caused by electrostatic interactions. Based on this hypothesis, we centrifuged the citrated rat blood to obtain PPP and BC (mainly red blood cells) (Shahidi et al., 2018). After mixed with poly(ATAC-*adj*-PEA) aqueous solutions, both PPP and BC formed a stable agglomerate. As anticoagulant citrate dextrose solution (ACD-A) was added to the blood for anticoagulation, citrate interrupts the blood clotting process by forming a calcium citrate complex, which disrupts the conversion of prothrombin to thrombin. (Ööpik et al., 2003). Therefore, we believe that the formation of “red clot” is mainly based on the electrostatic interactions between poly(ATAC-*adj*-PEA) aqueous solutions and negatively charged blood cells (mainly red blood cells), while “white clots” is based on the electrostatic interactions between poly(ATAC-*adj*-PEA) aqueous solutions and negatively charged plasma proteins and platelets. H&E staining result of agglomerate formed by blood cells and polymer solutions showed that the erythrocytes aggregated together, which indicated that poly(ATAC-*adj*-PEA) aqueous solutions can glue blood cells to form a blood gel.

2. The injectability of poly(ATAC-*adj*-PEA) aqueous solutions

In clinical applications, injection force is an important parameter for liquid embolic agents, which not only determines the suitability of the agent for clinical use but is also related to embolization treatment (physician administration and treatment effect) (Avery et al., 2016). The injection force can be affected by many factors, including the properties of the liquid agent (e.g., concentration and viscosity), injection rate, type of syringe and microcatheter, and injection conditions (e.g., air, liquid, or tissue) (Cilurzo et al., 2011; Vo et al., 2016). Injection force

should be sufficiently low to be usable by physicians. According to a review paper by Hu et al., the maximum forces of a typical injection applied by a male and a female are ~95.4 and ~64.1 N, respectively (Hu et al., 2019). Therefore, each polymer concentration tested in this study required comfortable forces between 4 and 10 N, which meets the basic requirements for a liquid embolic agent. In our research, we firstly replicated the injection process by injecting a polymer solution through 32-gauge clinical needles (I.D. 0.26 mm). The results showed that poly(ATAC-*adj*-PEA) aqueous solutions can be smoothly injected through needles with a relatively low force. However, Polymer concentration and injection rate significantly affected the injection force, which first increased and then plateaued in all tests. A high polymer concentration leads to a high solution viscosity, resulting in a large injection force. Meanwhile, high injection rates require a high injection force, which agrees with Poiseuille's law for fluid flow in tubes (Bruus, 2008). Moreover, we imitate clinical treatment situation by injecting polymer solution mixed with a contrast agent (tantalum powders, 0.25 g/mL), clinical microcatheters (I.D. 0.43 mm, length 150 cm), whose port was immersed in the citrated blood bath was measured. Then the injection was set by a program with 7-second injection and 3-second pause, repeated for four times. The results showed that injection force of polymer solutions increased to same level during injection and decreased during the pause in each cycle while injection force of Onyx 18 continually increased after each pause. All these results indicated that precipitated polymers partially blocked the microcatheter port, but polymer solutions didn't block the port. According to this, we considered that poly(ATAC-*adj*-PEA) aqueous solutions can be well used in clinical.

3. Adhesiveness of poly(ATAC-*adj*-PEA)-based blood gel to polyethylene tube

Adhesion to catheters is another factor need to be considered when developing new embolic agents. Fast polymerization of some LEAs may lead to adherence of catheter to vessel walls, which may increase the risk of hemorrhage when extracting catheter from vessels (Vollherbst et al., 2022; Higashino et al., 2021). Onyx, although was recognized as a non-adhesive embolic agent, there still some reports of catheter adhesion (Kim et al., 2021; Siekmann et al., 2005; Senturk, 2015). The embolization mechanism of our polymer solutions is the formation of blood

gel by electrostatic interactions between polymer solutions and blood contents. Therefore, it is necessary for us to confirm the adhesiveness of blood gel formed by polymer solutions. According to the *in Virto* traction test, low traction force of poly(ATAC-*adj*-PEA)-based blood gel was confirmed, which was much lower than that of Onyx 18 and similar to that of normal saline group. These results indicated that the blood gel formed by poly(ATAC-*adj*-PEA) aqueous solutions showed no obvious adhesion to plastic tubes, thereby considerably reducing the risk of hemorrhage in the traction process.

4. The biocompatibility of poly(ATAC-*adj*-PEA) aqueous solutions and poly(ATAC-*adj*-PEA) aqueous hydrogel

As a biomaterial for use in the body, good biocompatibility is very important. Not only should the material be non-toxic, but it should also not cause a strong immune stress response when applied to the body. In this research, we implanted poly(ATAC-*adj*-PEA) aqueous solutions as well as a poorly flowing entangled a disc-shaped poly(ATAC-*adj*-PEA) aqueous hydrogel (Ø5 mm, thickness 1 mm) under the dorsal skin for 7 and 28 days for biochemical tests and histological analyses of skin tissue. And the polymer hydrogels were further buried for 50 days to confirm biodegradability of polymers. The results of biochemical tests and historical analyses showed no obvious inflammation around the incision in either the polymer or hydrogel groups, demonstrating the safety and low toxicity of the poly(ATAC-*adj*-PEA) aqueous solution and hydrogel in rats. Moreover, the result of 50-day hydrogel subcutaneous implantation showed the hydrogel volumes shrank but the shapes did not change, indicating good stability of the polymers in the body. All these results of biocompatibility tests confirmed that poly(ATAC-*adj*-PEA) polymers are biologically well adapted and do not degrade over time in the body.

5. *In vivo* embolization capacity of poly(ATAC-*adj*-PEA) aqueous solutions

As we have confirmed the ability to aggregated blood contents *in vivo*, we have to verify whether the newly synthesized material can realize embolization (non-target) *in vivo*. Therefore, we firstly injected 0.1 mL of 40 mg/mL poly(ATAC-*adj*-PEA) aqueous solutions into right femoral artery of rats. Gradually darkened skin and immobile right hindlimb indicated

successful embolization reached by poly(ATAC-*adj*-PEA) solutions. Post-surgery H&E staining results showed occlusion clots left in vessels. The observation of red blood cells and nuclei of white cells implied the clot formed by the aggregation of blood contents.

6. In vivo post-surgery radiopacity of poly(ATAC-*adj*-PEA) aqueous solutions mixed with tantalum powders

Radiopacity is very important for embolic materials for position confirmation both intraoperation and post operation. However, like most polymers, our polymers are transparent and radiolucent. Therefore, we have to mix polymer aqueous solutions with tantalum powders in order to confirm the location of polymers after surgery. According to the preliminary experiments, we set the concentration at 0.25 g/mL. At first, the mixture of polymer solutions and tantalum powders was injected in a single shot (2–3 μ L) into the right Femoral artery of rats, and CT scanning was conducted at 3 h after injection. CT results indicated that embolization resulted by one-shot injection only occurred near the injection site instead of flowing far forward with the blood. Then, 0.05 mL of the mixture was injected into the rats' right femoral artery, which was subsequently CT scanned at 45 min after injection. The CT results showed that the mixture had good fluidity and could completely occlude the arteries of the right hindlimb. Next, to verify the reproducibility of this approach, we have repeated the rat femoral artery injection experiment by using 10 rats (20 hindlimbs in total), among which, 10 hindlimbs were injected with P(ATAC-*adj*-PEA) aqueous solutions (40 mg/mL, $\sim$ 10 μ L), another 10 hindlimbs were injected with normal saline water ($\sim$ 10 μ L) as a control. All rats were sacrificed and underwent CT scanning of hindlimbs on the 7th day after injection. All the hindlimbs became dark and tissue became necrotic over time. CT results confirmed existence of polymer mixture in all the Femoral arteries of experimental group which indicated that all these arteries were occluded by the blood gels. In contrast, the hindlimbs injected with saline were healthy. According to all these results, we confirmed the following things. Firstly, the problem that polymer solutions are radiolucent can be solved by mixing with tantalum powders. Secondly, polymer solutions can occlude artery by aggregating blood contents into blood gel, and this approach is able to be replicated. Besides, the embolization based on gluing blood

substances can be controlled by adjusting the injection volume and injection speed of poly(ATAC-*adj*-PEA) aqueous solutions. Thirdly, after mixing with tantalum powders, poly(ATAC-*adj*-PEA) (actually the blood gel formed by mixture of poly(ATAC-*adj*-PEA) aqueous solutions and tantalum powders) can be confirmed even on the 7th day after injection. In summary, this approach is considered to meet the requirements for future targeted treatment with the aid of X-rays.

7. Limitations of this research

Although we provided a new conceptual strategy for endovascular embolization, there are still some limitations in this research.

First, we only test poly(ATAC-*adj*-PEA), one of poly(cation-*adj*- π), during this research. Theoretically, all types of poly(cation-*adj*- π) own similar chemical structures, therefore they also share common chemical characteristics. In this research, all types of poly(cation-*adj*- π) we tested in this research showed the ability to react with blood, we didn't arrange further comparison experiments among these four tested poly(cation-*adj*- π) aqueous solutions. Besides, we only measured three concentrations, and mainly focus on the concentration of 40 mg/mL in this research. We didn't compare the subtle differences among different concentrations.

Second, due to the limitation in the use of experimental animals, we only tested the biocompatibility, stability and in vivo embolic effects on small animals. It may be further verified the biocompatibility, stability and in vivo embolic effects of this newly synthesized materials by medium or large animals.

Third, we only performed non-targeted in vivo embolization experiments on small animals because of the equipment limitations. The embolization effect of this newly synthesized polymer can be further confirmed if we can perform clinical embolization on cerebral disease models like cAVM in medium or large animals.

Finally, although we mixed polymer solutions with tantalum powders for radiopacity in this research, the use of tantalum powder will cause artifacts, which will affect clinical treatment. Besides, the same with Onyx, which is mixed with tantalum powder for radiopacity, the preparation period will become longer because of the use of tantalum powders. We hope we

can directly add substances like Iodine to this polymer which not only dissolve the problem of artifacts but also can reduce the preparation time.

Conclusion

1. Poly(cation-*adj*- π) aqueous solutions were able to react with whole blood and form a stable clot in a short time and the rheological results of the clot showed that it was a solid-like gel.
2. Poly(cation-*adj*- π) aqueous solutions could aggregate blood cells into a gel indicated that the aggregation was formed by electrostatic interaction.
3. Not only the polymer aqueous solutions but also the mixture of polymer aqueous solutions and tantalum powder, could smoothly pass-through clinical needles and microcatheters.
4. Blood gel formed by polymer solutions and blood showed low adhesiveness to microcatheter, compared to the clinically used Onyx.
5. Both poly(ATAC-*adj*-PEA) aqueous solutions and poly(ATAC-*adj*-PEA) aqueous hydrogel showed well biocompatibility and stability.
6. In vivo vascular embolization experiments demonstrated the ability of the polymer aqueous solution to react with blood to form a blood gel and successfully embolized the vessels. The polymer-tantalum mixture was also able to be visualized in postoperative CT to confirm the embolic location of the polymer after surgery.

In contrast to existing liquid embolic agents, whose embolization is based on the solidification of exotic materials, the water-soluble polymers bearing adjacent cation–aromatic sequences can glue negatively charged blood substances into a gel-like material through electrostatic interactions in normal saline water, whereas common polycations cannot form these interactions.

The poly(cation-*adj*- π) aqueous solution is expectant as a liquid embolic agent, owing to its easy delivery through a microcatheter with low injection force coupled with biocompatibility and controlled embolization. There are still some points need to be solved in future studies. First, the radiolucency of poly(cation-*adj*- π) aqueous solutions, which requires the addition of contrast agents (e.g., tantalum powders) for fluoroscopic visualization, increasing the preparing time as well as the existence of artifacts of CT images. Therefore, one of the future studies is to bound iodine component to polymers chemically. This will not only produce less the artifacts on CT but also reduce the preparing time compared to current conditions (mixed with tantalum

powders). Second, medium or large animal models, such as porcine aneurysm model will be conducted to further confirm the embolic effect of this newly synthesized polymer aqueous solutions. At the same time, medium or large animal models will also be used for confirming long-term (more than 6 months) biocompatibility of this new material, which small animal cannot. Good laboratory practice (GLP) tests will also be necessary in future studies.

In conclusion, this work is still in the early stages of research, but it provides a new idea for the realization of vascular embolization and could serve as a new direction for the development of the next generation of waterborne embolic agents.

Acknowledgements

This thesis is the culmination of a long and arduous journey that I have only been able to make as a result of dedicated support I have received from so many people along the way.

My first sincere appreciation goes to Professor Miki Fujimura of the department of Neurosurgery, Hokkaido University Graduate School of Medicine, for providing me with the opportunity to conduct this research. Then, my heartfelt thanks go to my supervisor Dr. Toshiya Osanai for his consistent and helpful support throughout my PhD studies. Next, I felt great honor that I have the opportunity to work with all the researchers from Institute for Chemical Reaction Design and Discovery (WPI-ICReDD), Hokkaido University and Faculty of Advanced Life Science, Hokkaido University. Among them, deepest and sincerest gratitude go to Dr. Hailong Fan, for his patience, help, and academic processions. Furthermore, I am grateful to all the researcher from Department of Forensic Medicine, Faculty of Medicine, Hokkaido University, for their help on animal CT scanning of my research.

I would like to express my gratitude to Ms. Rika Nagashima, research assistant, as well as all the secretaries of department of Neurosurgery. I am also very grateful to my parents, all my family members and my friends who are far away from Japan. Although we haven't seen each other for almost 3 years because of the COVID-19, their encouragement has always been with me. Finally, I am grateful to my boyfriend for his unwavering support. He never stopped encouraging me stood by me through all the hardships I had endured.

I am also grateful to Park Yong Koo Scholarship Foundation and Hokkaido University DX Doctoral Fellowship, for financial support which allowed me to concentrate on my studies and complete my thesis.

Finally, this research was supported by JSPS KAKENHI grant number JP17H06144, JP17H06376, JP21K14676, JPMJSP2119. The Institute for Chemical Reaction Design and Discovery (WPI-ICReDD), which was established by the World Premier International Research Initiative (WPI), MEXT, Japan. The authors thank Mr. Yoshiyuki Saruwatari at Osaka Organic Chemistry Co., Ltd., Japan, for providing the chemicals.

Disclosure of conflict of interest

The author declares no conflict of interest.

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