J Mater Sci: Mater Med (2009) 20:967–974
DOI 10.1007/s10856-008-3632-x

Fast thermoresponsive BAB-type HEMA/NIPAAm triblock copolymer solutions for embolization of abnormal blood vessels

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Received: 29 August 2008 / Accepted: 16 October 2008 / Published online: 20 November 2008

Abstract Thermoresponsive BAB-type HEMA/NIPAAm triblock copolymers (A = NIPAAm, B = HEMA) were prepared by atomic transfer radical polymerization (ATRP). BAB1-6 with shorter PNIPAAm blocks failed to form stable gel; while a relatively stable gel could be achieved by BAB1-8 with longer PNIPAAm blocks when copolymer aqueous solution was heated up. Introducing radiopaque agent (RA) was shown to slightly increase the transition temperature and gelation time, but the gelling ability was strengthened due to slightly weakening dehydration of copolymer in the mixture of water and RA. BAB1-8 aqueous solution about 5 wt% in the presence of RA was demonstrated to successfully occlude the cerebral rete mirabiles (RMs) and renal arteries of pigs. Within 3-month surgery, no recanalization was observed and the embolized kidney shrank considerably. Histological assay of embolized kidney demonstrated interstitial fibrosis and calcification as well as the thickening of renal small artery. This temperature sensitive copolymer with well-defined architecture holds a great potential as an embolic agent for treating arteriovenous malformations (AVMs) and renal disease due to the design flexibility of ATRP.

1 Introduction

Arteriovenous malformations (AVMs) are masses of abnormal blood vessels which may occur within the brain and other parts of the body. They consist of snarled tangles of arteries and veins. The goal of treating an AVM is to occlude the communicating channels of the nidus to prevent the enlargement of the collateral circulation from supplying the nidus with blood flow. In the case of AVM embolization, an interventional catheter is threaded through a small incision in the groin to the site of the AVM. Various materials, called embolic agents, can be injected into the abnormal blood vessels through the catheter, to completely close them. These embolic agents include tiny particles (microspheres) [1], tiny platinum coils [2], or a liquid glue [3]. However, the embolic agents currently used have several shortcomings, for example, it is difficult for solid microspheres to form dense packing, making it unfit for filling into rete mirabiles (RMs) (abnormal collection of tangled blood vessels, glomeruli of the kidney). Although liquid embolic agents such as cyanoacrylates and Onyx are clinically successful in treating AVMs, there still remain several problems like the organic solvents involved, adhesiveness to microcatheter and suboptimal handling at the time of surgical resection [4].

Temperature sensitive polymer aqueous solutions are able to reversibly change sol⇔gel states while temperature is below or above lower critical solution temperature (LCST). The flowable polymer sol may be conveniently injected into complicated shaped defects or rete mirabiles at lower temperature and rapidly gels in situ at body temperature with minimally invasive surgery [5]. Moreover, the polymer aqueous solution contains no organic solvents; thus they are ideal embolic agents for treating AVMs [6]. In spite of fast increase in the output of thermoresponsive polymers for theoretical purpose and biomedical engineering applications [7–18], the study on constructing thermoresponsive liquid embolic agents is surprisingly scarce in literature [5, 19, 20]. Recently, Vernon et al.

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[21–23] claimed to develop poly(NIPAAm-co-cysteamine) and poly(*N*-isopropylacrylamide-co-acrylic acid) copolymers for endovascular embolization. But they only reported on gelation properties, and suggested their possible application as embolic agents.

In our previous work [20], we synthesized thermosensitive *N*-isopropylacrylamide-*N*-propylacrylamide-vinyl pyrrolidone(PNINAVP) terpolymers which successfully occluded the RMs of swine in the presence of radiopaque agent Iohexol. It is of note that the PNINAVP random copolymers obtained by conventional polymerization technique lacked a precise molecular architecture and played an embolic role by thermally induced precipitation. Lately, we synthesized thermoresponsive ABA and BAB type triblock copolymers [A = *N*-isopropylacrylamide(NIPAAm), B = 2-hydroxyethyl methacrylate(HEMA)] by ATRP [24]. The thermoresponsive behaviors of ABA and BAB NIPAAm/HEMA copolymers aqueous solution were shown to be dependent on the order of block, and they underwent gelation via the interlocking of branch and flower micelles, respectively, at an elevated temperature. Moreover, BAB copolymer solution was transparent at a temperature below LCST due to more stable micelles formed. As for symmetrical BAB triblock copolymer (B as a hydrophobic block), the micellization and gelation have been investigated both theoretically and experimentally [25–27]. One interesting feature is that BAB copolymer in water tends to gel due to the formation of physically crosslinked network from interconnected micelles at higher concentration, which is distinct from self-assembly of AB diblock copolymer. This thermogelling of symmetric triblock copolymer portends a great potential as liquid embolic agent. In this work, we, for the first time, reported on the application of temperature sensitive injectable BAB triblock copolymer prepared by ATRP for the embolization of abnormal vessels of brain and kidney.

2 Materials and method

2.1 Materials

N-Isopropylacrylamide (NIPAAm, Aldrich Chemical Co.) was purified by recrystallization in hexane and dried under vacuum at 25°C. 2-Hydroxyethyl methacrylate (HEMA, 97%, Aldrich Co.) was treated with disposable inhibitor-removal column (Aldrich) to remove inhibitor. 1,4,8,11-tetramethyl–1,4,8,11-tetraazacyclotetradecane (Me4Cyclam) (98%, Fluka Co.), copper(I) chloride (Aldrich Chemical Co.), and the bifunctional atom transfer radical polymerization (ATRP) initiator, diethyl-meso-2,5-dibromoadipate (DEDBA) (Aldrich Chemical Co.) were used without further purification. Iopromide, radiopaque agent, was purchased from Guangzhou Pharmaceutical Company, China. Methanol was used after distillation.

2.2 Synthesis of $HEMA_n$–$NIPAAm_m$–$HEMA_n$ copolymers

BAB-type copolymers prepared by ATRP were reported in previously published work [24]. The chemical formula of BAB is depicted in Scheme 1.

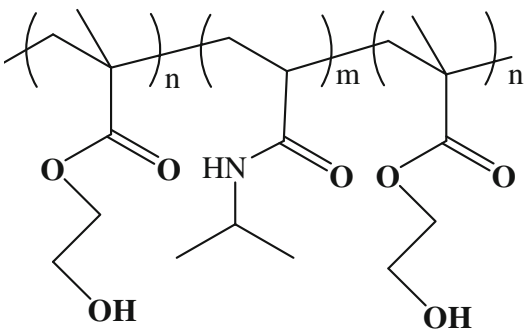
In this study, we provided two BAB copolymers, $HEMA_{40}$–$NIPAAm_{480}$–$HEMA_{40}$ and $HEMA_{40}$–$NIPAAm_{640}$–$HEMA_{40}$, which were coded as BAB1-6 and BAB1-8, respectively for simplicity of description.

Briefly, for synthesis of BAB1-6, DEDBA (9.1 mg, 0.025 mmol) and CuCl (4.9 mg, 0.05 mmol) dissolved in 2 ml of methanol were placed into a Schlenk tube and degassed by three freeze-thaw cycles. Then, NIPAAm (1.3578 g, 11.998 mmol, target degree of polymerization = 480) and Me4Cyclam (12.8 mg, 0.050 mmol) dissolved in 6 ml of methanol were added by a syringe under nitrogen and the reaction mixture was degassed by another three freeze-thaw cycles. After 6 h, degassed HEMA (0.2603 g, 2.0 mmol, target degree of polymerization = 40 for each block) in 2 ml of methanol was injected by a syringe. After 24 h the reactor was cooled by liquid nitrogen to terminate the reaction. The copolymer was collected by solvent evaporation and dialysis against deionized distilled water. In the same manner, BAB1-8 was also synthesized.

The molecular weights and the molecular weight distributions (GPC Waters 510/M32, THF as a mobile phase, monodisperse polystyrene as a standard): BAB1-6 ($\overline{Mn}$= 60,000, PDI = 1.13), BAB1-8 ($\overline{Mn}$= 57,700, PDI = 1.14).

2.3 DSC measurement

Thermal analysis was performed on a differential scanning calorimeter (NETZSCH DSC200F3). 10 µl of 5 wt% BAB1-6 or BAB1-8 copolymer aqueous solution was hermetically sealed in an aluminum pan. Heating scans



Scheme. 1 Chemical formula of $HEMA_n$–$NIPAAm_m$–$HEMA_n$

were recorded in the range of 20–45°C at a scan rate of 5°C/min. Deionized water was used as a blank reference. The mixtures of BAB1-6 or BAB1-8 with radiopaque agent in an equal volume ratio, which was separately denoted as BAB1-6R and BAB1-8R, was detected in the same way. Note that in this work, BAB1-6 and BAB1-8 remained the same concentration in the mixture as its blank copolymer.

2.4 Rheological properties

The oscillatory shear measurements were performed on a stress-controlled rheometer RELOGICA INSTRUMENTS AB using a Bob-Cup 25 geometry. The rate of temperature increase was controlled at 0.5°C/min by a computer-programmable circulator with a precision of ±0.05°C. The storage moduli (G′) and loss moduli (G″) of the copolymer solutions or the mixtures with radiopaque agent were measured as a function of temperature, at a constant angular frequency of 0.2 Hz.

2.5 In vivo embolization and histological assay

Ten Chinese mini-pig of both sexes weighing 25–30 kg were used. 5 wt% BAB1-8 aqueous solution was formed by mixing 10% BAB1-8 with equal volume of radiopaque agent, Iopromide, and the mixture was injected into RMs or a renal artery of pig separately through a Prowler14/Magic1.8 F and 4F microcatheter based on the protocols of animal experiment reported in our previous paper [20]. In this experiment, 1 ml and 2–3 ml of embolic agent was separately injected into RMs and renal artery at a speed 60–100 s/ml. Dynamic angiography was performed till satisfactory embolization was achieved. The angiographic images were recorded prior to embolization, immediately, 15 days, 2 months and 3 months after embolization. It is noted that at each time point, we injected fresh contrast agent again for imaging. All procedures reported here were in accordance with China National Animal Law on the use of laboratory animals.

Three swines were sacrificed by pentobarbital overdose immediately, 15 days and 3 months after the embolization. The embolized RM removed surgically was macroscopically examined and then fixed in 10% formalin at 36.5°C because the copolymer liquefies easily at temperature below LCST. Thin tissue sections were prepared and stained with hematoxylin–eosin (HE) for the histological examinations.

3 Results and discussion

In designing temperature sensitive BAB triblock copolymers for endo-vascular embolization, HEMA was deliberately incorporated into the macromolecular chains, aiming to elicit the reaction of complement system in the vicinity of abnormal vessels with hydroxyls [28, 29], which may cause thrombus, and accordingly facilitates long-term stable embolization that will be verified in the section of in vivo embolization. Additionally, the basic premise of thermoresponsive liquid embolic agent should be that the aqueous solution of polymer is in injectable sol state at room temperature; while it is capable of quickly gelling once temperature is above body temperature. For BAB HEMA/NIPAAm triblock copolymer, we found 1 wt% solution failed to form gel while temperature is up to 37°C. Whereas 10 wt% solution is very viscous, worsening the injectability, which could lead to the blockage of long microcatheter in vivo. Thus, in this experiment, 5 wt% concentration was used considering its better injectability as well as gelling capacity. Figure 1 shows DSC curves of 5 wt% BAB copolymer aqueous solutions and their mixture with radiopaque agent. The peak temperatures (Tp), transition maxima of BAB1-6 and BAB1-8, are 34.1 and 34.3°C, respectively. It is evident that BAB solution remains almost constant in transition temperature with the same chain length of PHEMA. A noticeable phenomenon is that Tps of BAB1-6R and BAB1-8R are increased up to 36.1 and 35.7°C, respectively. However, the transition is still below body temperature. We cannot give a complete explanation of Tp increase with incorporation of radiopaque agent here. Nevertheless, we can judge that the dehydration of BAB copolymer in the mixture of water and radiopaque agent was slightly impeded.

The dynamic viscoelastic properties are very important for temperature sensitive embolic liquids. It should be an injectable viscous fluid at low temperature for easy injection to an AVM through a thin, long microcatheter. After reaching the abnormal vessels, the fluid should form stable

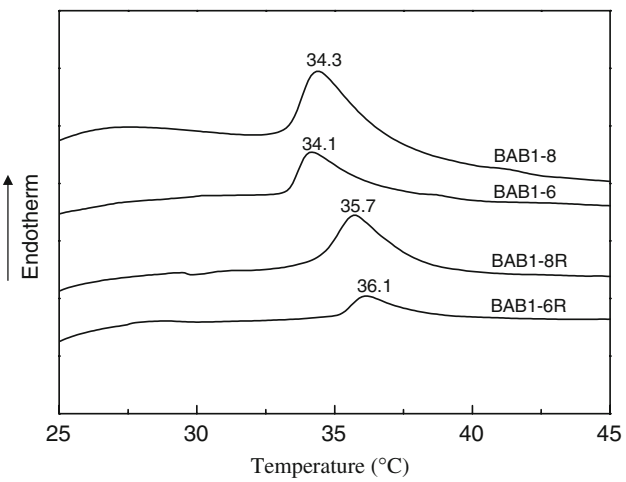


Fig. 1 DSC heating thermograms of 5 wt% BAB copolymer aqueous solutions and their mixtures with radiopaque agent. Peak temperatures are labeled in the figure

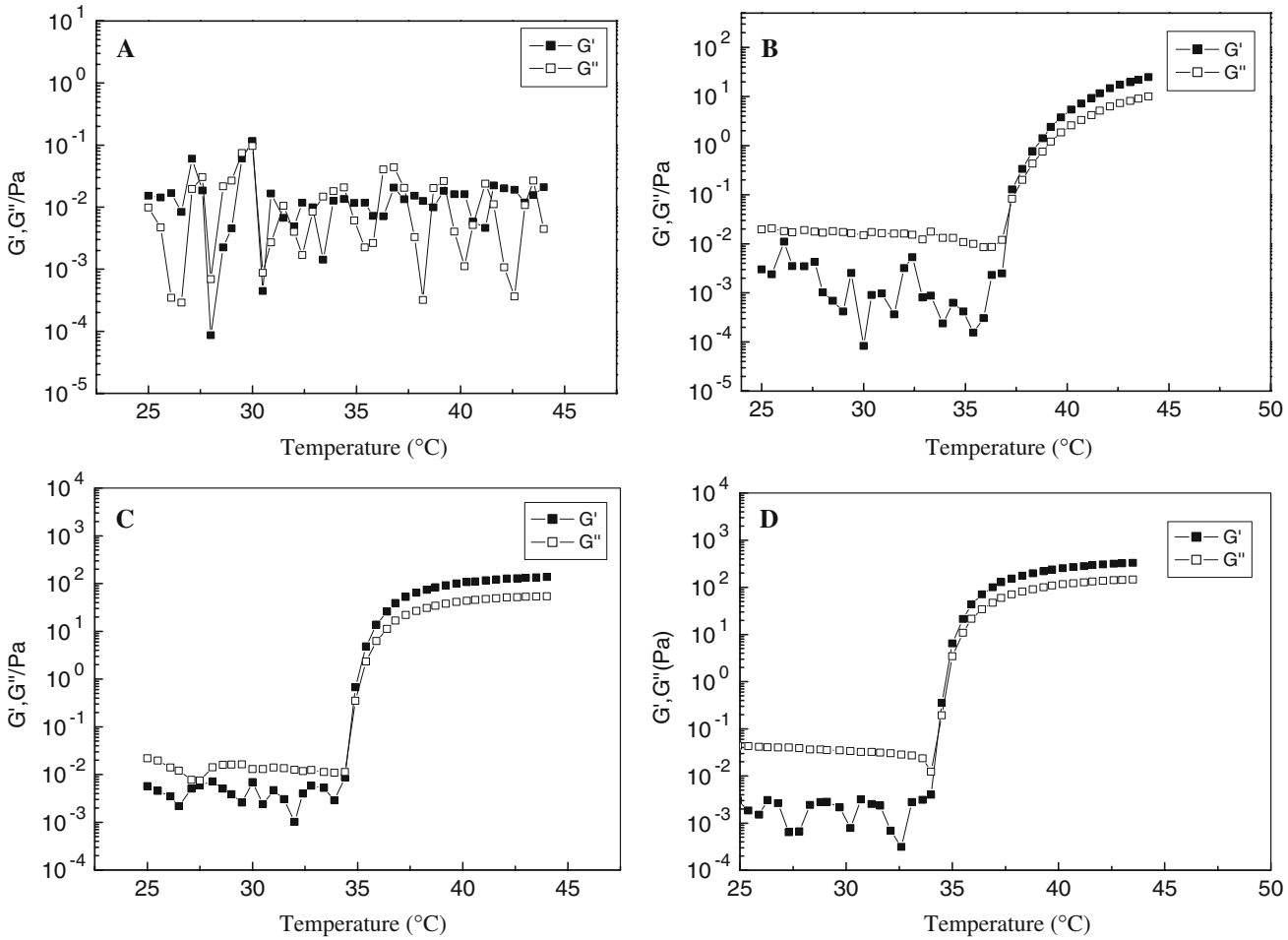


Fig. 2 Variation in G′ and G″ as a function of temperature for 5 wt% BAB1-6 (**a**), 5 wt% BAB1-6R (**b**), 5 wt% BAB1-8 (**c**), 5 wt% BAB1-8R(**d**)

gel to adhere to arterial bed for the effective embolization. Hence, the gelling embolic agent ought to behave as a solid with sufficient scission moduli. Herein, we checked the variation in moduli of 5 wt% BAB1-6 and BAB1-6R solutions (Fig. 2a, b). For 5 wt% BAB1-6 solution, we can see a severe jump of moduli during heating, and no evident change in G′ and G″ in the selected range of temperature, suggesting BAB1-6 solution is unable to form firm gel due to the incorporation of more of hydrophobic HEMA moieties. The jump of moduli is possibly originated from the dynamic entanglement and disentanglement of polymer chains caused by oscillatory shear. Interestingly, incorporating radiopaque agent significantly reduces the fluctuation of moduli, in particular for G″ and post-transition region. Moreover, above 36.3°C, there appears an abrupt increase in moduli, and G′ starts to overtake G″, which hints the occurrence of gelation. As mentioned above, the addition of RA deters the dehydration of copolymer to some extent, which aids in the gelling of embolic agent. However, no plateaus of G′ and G″ appear over experimental temperature range, suggesting the phase transition is incomplete and the weak gel formed is unstable. In comparison, for BAB1-8 system, the variation of moduli becomes relatively stable, and at temperature above 34.4°C, a sharp rising of moduli is observed with G′ exceeding G″ (Fig. 2c, d). Moreover, G′ and G″ approach a plateau around body temperature, which implies the formation of relatively stable gel. It should be pointed out that a qualitative description of modulus change during phase transition is merely presented here; we cannot give a quantitative comparison of moduli between BAB1-6 and BAB1-8 owing to the existence of fluctuation in the course of heating.

From the above analysis, we find that the block length of PNIPAAm strongly influences the thermogelling properties–longer PNIPAAm facilitates the gelation due to heat-triggered association of macromolecular chains. Figure 3 displays the optical images of sol⇔gel transition with addition of radiopaque agent. Note that in this study, the volume ratio of copolymer to radiopaque was kept at 1/1. It is clearly seen that BAB1-8 solution with inclusion of RA remains transparent at temperature below LCST; while temperature is raised above LCST, the solution formed gel.

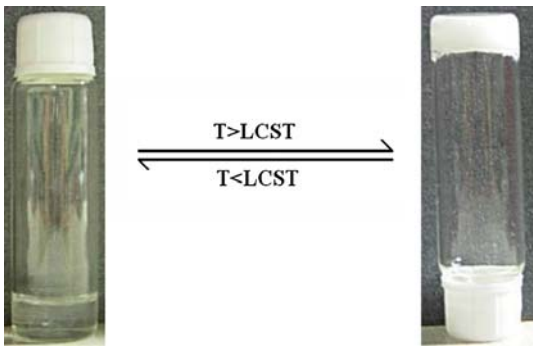


Fig. 3 Optical images of sol⇔gel transition with inclusion of radiopaque agent. The concentration of BAB1-8 is 5 wt% and the volume ratio of BAB1-8 to radiopaque agent is 1:1

One should bear in mind that apart from the above properties, the gelation time of an embolic agent is critical for successful embolization. In light of the potential of 5 wt% BAB1-8 as an embolic agent, we measured the gelation time using inverted test tube method. As shown in Table 1, the gelation time of BAB1-8 is under 1 min—that is an ideal embolization and injection time. Based on our experience on embolization [20], longer gelation time will lead to errant embolization due to the washing-away effect of bloodstream; too short gelation time tends to cause blocking of catheter, making handling difficult. It should be emphasized that the gelation properties of blank copolymer solution cannot be extrapolated to real embolic agent because the radiopaque agent loaded might influence the behavior of sol-gel of the final thermoresponsive embolic agent. Encouragingly, the gelation time with RA loading was just elongated to a less extent, but still within 1 min after encapsulating RA (Table 1).

Table 1 Gelation time of aqueous solution determined by inverted test tube Temperature (°C)

Sample	Gelation time(s)
BAB1-8	41.1 ± 3.2
BAB1-8R[a]	49.6 ± 2.4

[a] BAB1-8R denotes the mixture of BAB1-8 and radiopaque agent at volume ratio of 1:1

The BAB1-8R was injected through a microcatheter into pig's the desired cerebral and renal blood vessels to examine the effect of embolization. The angiographic images clearly display the presence of ascending pharyngeal arteries and RMs before operation (Fig. 4a). The results obtained immediately (b), 15 days (c), 2 months (d)

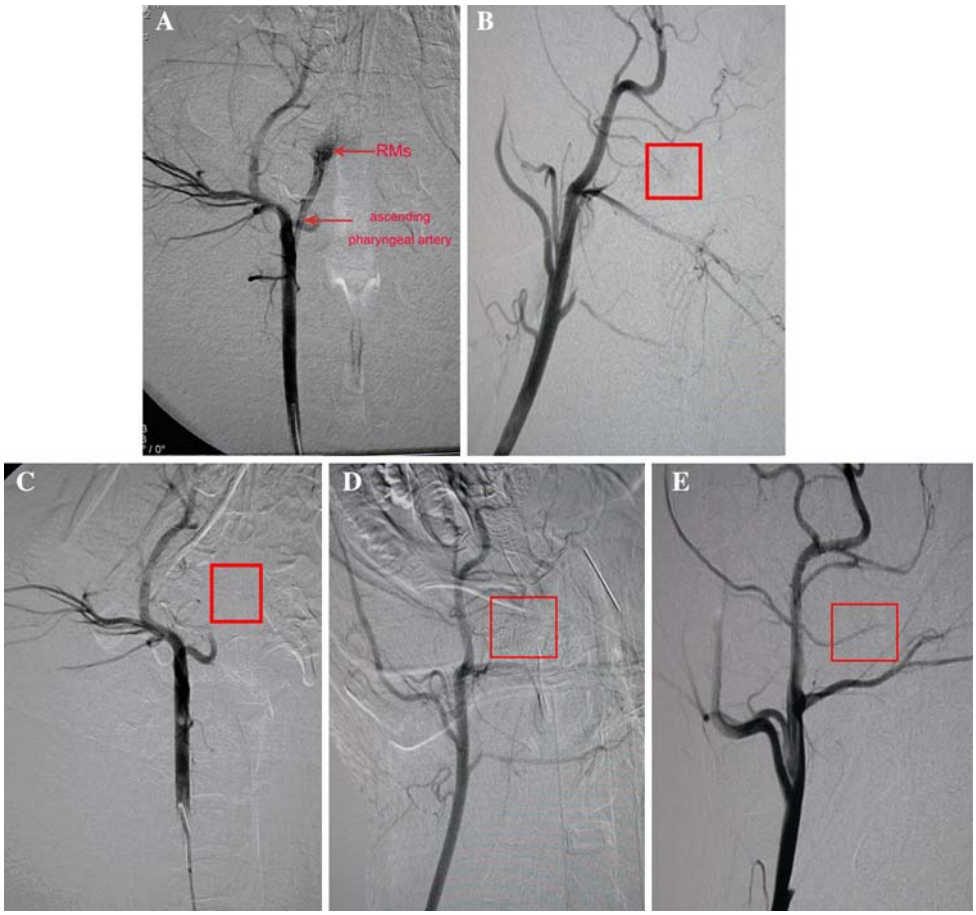


Fig. 4 Angiograms obtained before (**a**), immediately (**b**), 15 days (**c**), 2 months (**d**), 3 months (**e**) after embolizing the RMs of Chinese mini-pig with 5 wt% BAB1-8R

and 3 months (e) after embolization, show that they are invisible because of occlusion by BAB gelation.

Figure 5 demonstrates the angiographic images of embolized right kidney at several time points. As shown in the picture, the right renal artery and its peripheral branches were visible before injection of embolic agent (Fig. 5a). Likewise, the renal artery and vein disappear in angiographic pictures taken immediately, 15 days and 2 months after surgery(b, c, d), revealing the effective embolization. Reexamination at 90 days after surgery shows no recanalization (Fig. 5e). A notable feature is that compared to control left kidney, the embolized right kidney after embolization for 3 months shrank considerably (Fig. 6), which is due to the obstruction of blood supply caused by gelling copolymer. Histological assay of excised kidney sample obtained immediately after embolization reveals cloudy swelling renal proximal tubular epithelia and blurry boundary (Fig. 7a). The droplet degeneration of tissue is also observed (indicated by arrow, Fig. 7b). Fifteen days post-operative results show there appear renal tubular necrosis (Fig. 7c), thrombus of renal interstitial small artery (indicated by arrow, Fig. 7d), and kidney glomerulus (indicated by arrow, Fig. 7e). Partial glomerular mesangium hyaline degeneration is also present (Fig. 7f). As aforementioned, introducing PHEMA into copolymer may arouse blood clot due to the activation of complement system. Thus, the polymer gel combined with thrombus can be fixed into the vessel wall, synergically contributing to long term embolization.

At post-operative 3 month, we can observe the atrophy of pale kidney (Fig. 7g), glomerulosclerosis (Fig. 7h), interstitial fibrosis and calcification (Fig. 7i). The thickening of renal small artery wall and luminal narrowing accompanied with fibrinoid necrosis is clearly seen (Fig. 7j).

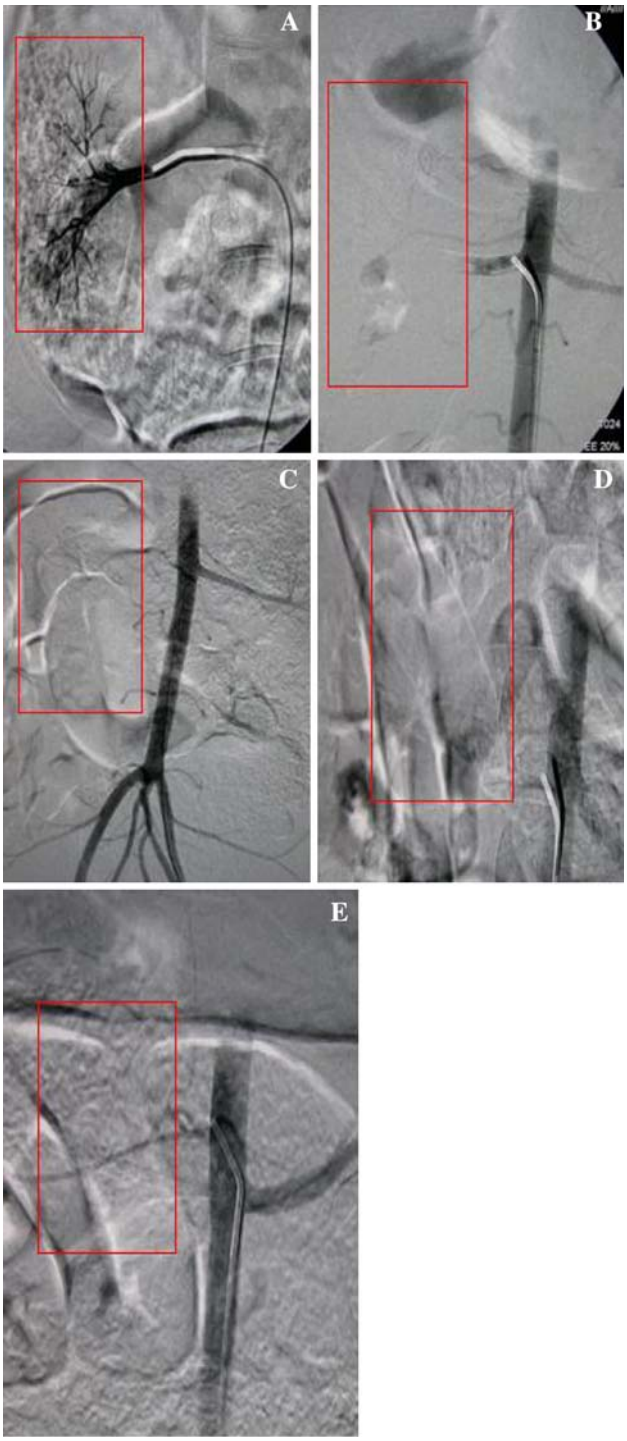


Fig. 5 Angiograms obtained before (**a**), immediately (**b**), 15 days (**c**), 2 months (**d**), 3 months (**e**) after embolizing the right kidney of Chinese mini-pigs with 5 wt% BAB1-8R

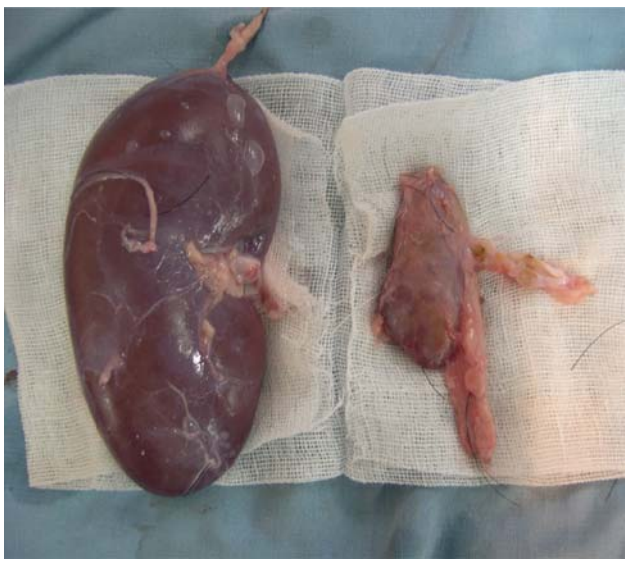


Fig. 6 Comparison of normal left kidney with embolized right kidney after 3-month surgery

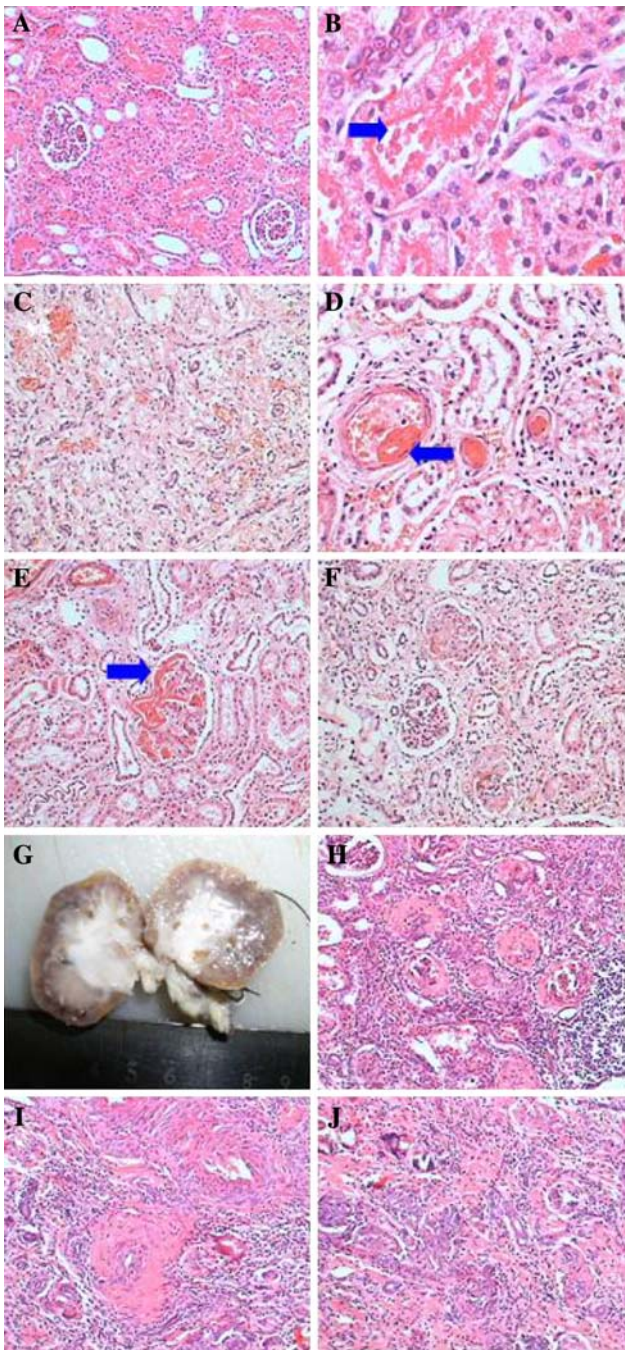


Fig. 7 Histological assay of excised kidney sample immediately (**a** ×100; **b** ×400), 15 days (**c** ×100; **d** ×200; **e**, **f** ×100), 3 months (**g**–**j** ×100) after embolization. Tissue excised was stained with Hematoxylin & Eosin (HE). Droplet degeneration (**b**), thrombus of renal interstitial small artery (**d**) and kidney glomerulus (**d**) was indicated by arrow

To further confirm the embolization effect, ascending pharyngeal artery and renal artery were excised. The pathological analysis reveals that BAB1-8R embolic agent is present in the embolized vessel lumen (Fig. 8), evidencing a stable embolization effect.

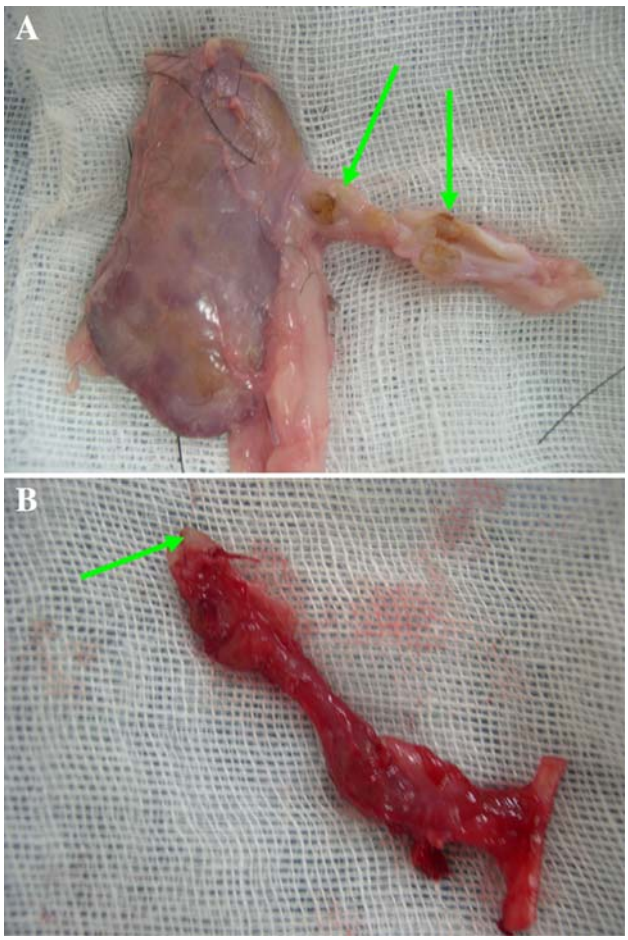


Fig. 8 Pathological analysis of the excised renal artery (**a**) and ascending pharyngeal artery (**b**) after 3-month embolization. Arrow indicates the presence of gelling BAB1-8R in vessel lumen

4 Conclusions

Thermoresponsive BAB-type HEMA/NIPAAm triblock copolymer in aqueous solution was capable of rapidly gelling at an elevated temperature. Incorporation of radiopaque (RA) could improve the gelling capability due to the weakening dehydration in the mixture. The mixture of 5 wt% BAB1-8 and RA was shown to effectively occlude the cerebral RMs and renal arteries of pigs. Within 3-month surgery, no recanalization was observed and the embolized kidney occurred to shrink significantly, suggesting this copolymer holds a great potential as an embolic agent for treating AVMs and renal disease by long-term embolization. The living radical polymerization technique can also be used to tailor-make a temporary embolic agent by introducing biodegradable blocks which will be degraded in vivo.

Acknowledgments The authors gratefully acknowledge the support for this work from the National Natural Science Foundation of China

(Grant 50573054), Tianjin Municipal Natural Science Foundation (07JCYBJC02800), and Program for New Century Excellent Talents in University.

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