Tumor Noninvasive and Target Embolization Therapy Platform by Intravenous Injection Based on Acidic Microenvironment-Responsive Hyperbranched Poly(amino acid)s

Dedai Lu,* Jiachen Wang, Yunfei Li, Yongyong Zhang, Lili Yu, Tingting Xu, Hongyun Guo, Yongdong Zhang, Xingdong Wang, Xiaoqi Wang, Gaojun Teng, and Ziqiang Lei

ABSTRACT: Transcatheter arterial embolization (TAE) has been widely applied in treatments of unresectable or hypervascular tumors, but the procedure of TAE is complicated possibly brings inherent risks. Here, inspired by pH-responsive drug delivery systems, a new method of noninvasive and target embolization therapy by intravenous injection was developed. This method is based on a type of acidic microenvironment-responsive hyperbranched poly(amino acid) (HPTTG) to avoid using catheterization and real-time image guidance angiography, simplifying the procedure, elevating compliance and general applicability of embolization therapy. The pH value of the sol-to-gel phase transition with decreasing pH of HPTTG was controlled by adjusting the ratio of acidic amino acids in copolymers. The results of the tumor-bearing animal experiment indicate that the HPTTG have an excellent target and embolic ability; they accumulate the most at the tumor site in 8 h postinjection. Blood vessels of the tumors were occluded, and the tumors were inhibited and necrotized in about 20 days. Therefore, it is expected that HPTTG not only can be used as novel embolic materials for efficient noninvasive embolization therapy of many solid tumors but also can be used as a multifunctional platform for combined theranostics, for example, combination with controlled release, thermal ablation, multimodal imaging, synergistic therapy, etc.

1. INTRODUCTION

Embolization therapy has been widely applied in clinical practices and is as important as medicine and surgical treatments for tumors. Transcatheter arterial embolization (TAE) or transcatheter arterial chemoembolization (TACE), the most commonly used procedure, has become the first-line treatment for unresectable or hypervascular tumors, such as hepatocellular carcinoma (HCC).1−4 TAE produces rapid effects, is easy to combine with other therapies, and is recommended as a gold standard for unresectable HCC.5−6

However, TAE (TACE) has many defects in clinical practice. First, viewed from the embolic materials, many materials have been used as embolic agents including cyanoacrylate glues, poly(vinyl alcohol) (PVA) microparticles, lipiodol, ethylene (vinyl alcohol) polymer and precipitant gels, and polymer microspheres, etc.7−11 Unfortunately, there are several hazardous drawbacks in their clinical application, for instance, adhesion to catheters, use of organic solvents, incomplete embolization and nontargeted embolization, vascular recanalization, toxicity, and difficulties in use.12−15

In situ gelling polymer systems, which can avoid the use of toxic byproducts or solvents, have received particular attention as novel embolic agents. The stimuli- or environment-responsive polymers, including temperature-sensitive poly(N-isopropylacrylamide) (PNIPAM) and pH-responsive polymers, have also been reported to be used for embolization therapy of HCC.5,6,16−19 However, many temperature-sensitive materials exhibit viscoelastic flow under constant and low-frequency stress; they may also clog within the long microcatheter once body temperature is reached. The lack of ionic moieties may limit the interactions with a broad range of therapeutic agents.17 In general, existing embolic materials have their advantages and shortcomings.10

Furthermore, the technique of TAE as well as its embolic materials have their own limitations. While TAE is most frequently used for HCC, it is highly dependent on the type and location of the tumor, different practice patterns, operator and technical variables, and trial design (end point selection, response assessment).20 The procedure of TAE is complicated and demanding, with one (or more) superselective catheterization requiring repeat contrast-injected real-time angiography throughout the procedure. There also are many potential risks of side effects and complications, including nontarget emboliza-

Received: April 24, 2020
Published: October 15, 2020

This is an open access article published under an ACS AuthorChoice License, which permits copying and redistribution of the article or any adaptations for non-commercial purposes.
tion, ischemic pain, tumoral hemorrhage, postembolization syndrome, damage of normal tissue and its function, toxicity of the high dosage contrast agent, etc.\textsuperscript{21−23}

Recently, many drug delivery systems based on pH-responsive polymers have been reported to be used in anticancer therapy.\textsuperscript{24−26} Solid tumors characteristically display higher levels of lactate production due to the anaerobic metabolism of glucose. A high rate of anaerobic glycolysis in solid tumors contributes to an acidification of pH to ∼6.7−7.2 in the tumor microenvironment due to lactate accumulation.\textsuperscript{27−29} When the pH-responsive polymer solution is injected into the extracellular acidic tissues of the tumor (pH ranges from 6.5 to 7.2), the polymers will change from liquid-solution to solid gel state. In order to minimize the risks and complications, avoid the usage of a microcatheter and real-time image guidance angiography, simplify the procedure, and elevate compliance and general applicability of embolization therapy, we developed the noninvasive embolization for solid tumors based on tumor

Table 1. Molecular Mass and Monomeric Unit Ratio of Poly(amino acid)s

| polymer   | $M_n^a$ (kDa) | $M_w^a$ (kDa) | PDI$^a$ | $M_n^b$ (kDa) | $M_w^b$ (kDa) | PDI$^b$ | I/Th:E:Ty$^c$ | I/Th:E:Ty$^d$ | $M'$ (kDa) | $\zeta$ potential (mV) | HD (nm) |
|-----------|---------------|---------------|---------|---------------|---------------|---------|--------------|--------------|------------|--------------------------|----------|
| HPTTG-6.8 | 23.24         | 52.48         | 2.25    | 20.43         | 27.78         | 1.36    | 1:80:50:100  | 1:51:38:75   | 24.9       | −16.42 ± 0.5             | 201 ± 15 |
| HPTTG-3.5 | 21.14         | 50.23         | 2.37    | 19.32         | 27.59         | 1.42    | 1:120:120:0  | 1:96:88:0    | 23.5       | −17.77 ± 0.5             | 198 ± 15 |

$^a$Molecular mass and PDI were calculated via light scattering. $^b$Molecular mass and PDI were calculated via GPC. $^c$Feed ratio of monomer. $^d$Polymer ratio of monomer observed by $^1$H NMR. $^e$Molecular mass calculated using the polymer observed ratio of monomers. I, Th, E, and Ty show the number of initiators, L-threonine, L-glutamic, and L-tyrosine, respectively.

Figure 1. (A) Schematic explanation of the mechanism of the gelation of HPTTG. Upon the change of environmental pH, a three-dimensional hydrogel network can be formed and thus block the targeted blood vessels. (B) Transmittance–pH curve of copolymers (A, HPTTG-3.5; B, HPTTG-5.1; C, HPTTG-6.8; D, HPTTG-10.0). (C) Time–mass loss curves of polymers. (D) CCK8 assay OD value of 578 cells which were cultured with the extraction media from HPTTG. (E) Cell proliferation of 578 cells.
microenvironment-gelatinized poly(amino acid)s, taking inspiration from pH-responsive drug delivery systems. Poly(amino acid)s were specially selected due to their biocompatibility, biodegradability, and diverse functionality. The pH value of the sol-to-gel phase transition with decreasing pH of poly(amino acid)s was controlled at 6.5–7.0 by adjusting the ratio of acidic amino acids in copolymers. It is expected that the poly(amino acid)s will undergo gelation transition after intravenous injection and reach the tumor site via blood circulation; thus, it is possible to achieve the noninvasive and target-embolization of solid tumors.

Specifically, the pH-responsive hyperbranched poly(amino acid)s, poly[l-threonine-b-(L-glutamic acid-ran-L-tyrosine)] (HPTTG) with a tunable pH-responsive value are prepared via ring-opening polymerization of L-amino acid N-carboxyanhydride (NCA), as is illustrated in Scheme S1. Due to its lower hydrodynamic volume, larger number of modifiable end groups, and better solubility of dendritic or hyperbranched polymers compared to linear analogues, the hyperbranched copolymer precursors were adopted in this study. Various properties, including the sol–gel phase transition, the cytotoxicity, and biodegradability, of the copolymers and its aqueous solution were examined. In addition, a mouse tumor was used to examine venous injectability and gullibility of HPTTG-based embolic formulations. Furthermore, H22 tumor-bearing mice and VX2 tumor-bearing rabbits were used to examine the ability of target embolic materials to form embolization hydrogels at the tumor site and inhibit the tumor growth.

2. RESULTS AND DISCUSSION

Hyperbranched polymers have a lot of advantages, especially for hyperbranched polyglycerol (HPG); it has excellent biocompatibility, higher transport capacity, good water-solubility, and multiple terminal groups for modification. Therefore, the hyperbranched poly[l-threonine-b-(L-glutamic acid-ran-L-tyrosine)] (HPTTG) is prepared by ring-opening polymerization (ROP) of NCA using amino-terminated hyperbranched polyglycerol as the initiator. The successful synthesis of HPTTG is verified by $^1$H NMR (Figures S1 and S2). Absolute number-average molecular weight was calculated using the integral area ratio of characteristic hydrogen of monomeric units. The average molecular weight and polydispersity index (PDI) of the HPTTG were determined by GPC (Figures S3 and S4) and light scattering (LS). As shown in Table 1, the calculated number-average molecular weight was approximately equal to the measured number-average molecular weight. Notably, the PDI (LS) is high because of the high tendency of association of polymer chains and the interference of the small molar mass fraction. $M_0$ obtained by LS was significantly higher than that obtained by GPC. This might because the average molar mass obtained by GPC is based on the hydrodynamic volume ($V_0$) of the polymer chains, and branched polymers have lower $V_0$ than their corresponding linear analogues with the same molar mass. This also shows that we successfully synthesized a compact, highly branched HPTTG. The particle size and surface charge of HPTTG-6.8 and HPTTG-3.5 were assessed. As is shown in Table 1, there is no significant difference in the surface charges of HPTTG-6.8 and HPTTG-3.5. The surface charge affects their biodistribution and clearance. The blood vessels have a negatively charged surface; negatively charged particles showed high accumulation at the tumor site, less hematotoxicity, and long residence in blood. Through the measurement of the particle sizes of HPTTG-6.8 and HPTTG-3.5, results show that the hydrodynamic diameters (HDs) of HPTTG-6.8 and HPTTG-3.5 are relatively close and are consistent with appropriate biological parameters. The general reason for the gelation of HPTTG may be due to the carboxyl groups and phenolic hydroxyl groups in the HPTTG side groups. When the pH value of the solution is above the $pK_a$ of carboxyl groups and phenolic hydroxyl groups, the carboxyl groups and the phenolic hydroxyl groups are ionized. When the pH value of the HPTTG solution decreases, the carboxyl groups and the phenolic hydroxyl groups are deionized; intramolecular hydrogen bonds are formed. The chain of HPTTG is shrunk, and the HPTTG segment, being a hydrophilic segment, allows it to wrap water molecules to form a hydrogel. The $pK_a$ value of the acidic or basic groups leads to the pH response changes of poly(amino acid)s, such as poly(l-glutamic acid) ($pK_a$ 4.1) and poly(l-tyrosine) ($pK_a$ 10.0). When hydrophilic or hydrophobic polymeric units or a second polyelectrolyte with desirable $pK_a$ value is introduced into the poly(amino acid) chains, a tunable pH-response poly(amino acid) will be obtained. The sol–gel state transition may be affected by a number of factors, including the concentration of the polymer in the aqueous solution and the block ratio of monomeric units, which have been studied and discussed in previous studies. Here, we explored the effect of concentration and block ratio on the gelation of HPTTG. In Figure 1B, the pH of the sol–gel transition of the HPTTG solution was determined by UV spectrophotometer. When the transmittance was less than 50%, the pH value of HPTTG was determined as the sol–gel transition pH value of HPTTG. In this study, we found that the sol–gel transition of HPTTG changed with the monomeric unit ratio of L-Tyr to L-Glu. The result showed that the pH-responsive value of copolymers is tunable from 3.5 to 10. When the units ratio was L-Tyr:L-Glu = 0:100, the sol–gel transition pH value was about 3.5 (HPTTG-3.5). When the units ratio was L-Tyr:L-Glu = 100:0, the sol–gel transition pH value was about 10.0 (HPTTG-10.0). When the units ratio was L-Tyr:L-Glu = 50:50, the pH-response was 5.1 (HPTTG-5.1). When the units ratio was L-Tyr:L-Glu = 58:18, the sol–gel transition pH value was about 6.8 (HPTTG-6.8). As we know, the pH value of normal tissue is 7.2–7.4, but the tumor tissue microenvironment is acidic (below pH 6.8). Since the pH-response of HPTTG-6.8 is similar to the pH of the tumor acidic microenvironment, HPTTG-6.8 can be used to form hydrogels in the tumor acidic microenvironment and then embolized the blood supply artery of the tumor with minimal effect on the normal tissue in a normal physiological environment. Therefore, it can be applied in the field of tumor embolization treatment.

The enzyme degradation reaction was used to measure the degradation property of HPTTG. As shown in Figure 1C, under the action of the protease, the mass loss of HPTTG-6.8 was more than 70% after 18 days. Therefore, the degradation rate of the polymer conforms to the enzymatic degradation performance requirements of general biological materials, indicating that HPTTG has excellent biodegradability.

Hemocompatibility is one of the most important standards to evaluate safety for biomaterials. In this study, hemolysis and coagulation studies were used to evaluate hemocompatibility of HPTTG: calculated prothrombin time (PT) and activated partial thromboplastin time (APTT). HPTTG at a maximum mass percent of 1% showed 2.57 ± 0.3% hemolysis. The standard range of PT is 11–14 s; the standard range of APTT is 27–40 s. Compared to normal values, PT of HPTTG had no obvious change, and APTT increased to 55 s.
The biocompatibility of HPTTG was investigated by measuring the cytotoxicity of HPTTG to 578 cells by the CCK8 assay. As shown in Figure 1D,E, when the HPTTG concentration was 5 mg/mL, the survival rate of 578 cells was greater than 80%; when the concentration was 20 mg/mL, the survival rate of 578 cells was greater than 70%, indicating that HPTTG has good biocompatibility and has low toxicity to cells itself. There was also no significant difference between the experimental group and the control group. This indicates that HPTTG has excellent biocompatibility.

Figure 2. Efficient accumulation in tumors. (a) Cy5.5-HPTTG-6.8 was injected into the tail vein of the hepatic tumor model of mice. (b) Cy5.5-HPTTG-6.8 was injected into the tail vein of the right forelimb tumor model of mice. (c) Cy5.5-HPTTG-3.5 was injected into the tail vein of the right forelimb tumor model of mice. (A) Fluorescence images of mice at 4, 8, 12, 24, 48, and 72 h postinjection of Cy5.5-HPTTG. (B) Fluorescence image of hepatic tumor mouse tissues and subcutaneous tumor mouse tissues 72 h postinjection. (C) Distribution of FITC-HPTTG-6.8 in the tumor site. (D) Quantification of the Cy5.5-HPTTG-6.8 biodistribution in hepatic tumor model mice. (E) Quantification of the Cy5.5-HPTTG-6.8 and Cy5.5-HPTTG-3.5 biodistribution in right forelimb tumor model mice. The data are mean ± SD (n = 3); **, P < 0.01. (F) Biodistribution of 131I-HPTTG in H22 tumor mice. (G) SPECT and SPECT/CT photograph of the rabbit. (H) DSA images of the hepatic tumor.

The biocompatibility of HPTTG was investigated by measuring the cytotoxicity of HPTTG to 578 cells by the CCK8 assay. As shown in Figure 1D,E, when the HPTTG concentration was 5 mg/mL, the survival rate of 578 cells was greater than 80%; when the concentration was 20 mg/mL, the survival rate of 578 cells was greater than 70%, indicating that HPTTG has good biocompatibility and has low toxicity to cells itself. There was also no significant difference between the experimental group and the control group. This indicates that HPTTG has excellent biocompatibility.
The purpose of embolization is to reduce the blood supply around the tumor site, cut off the nutrient supply of the tumor, and finally inhibit its growth.\textsuperscript{43} We first examined whether HPTTG could accumulate at the tumor site, exploring its rate of accumulation at the tumor site. Biological studies were conducted in animal models. In this study, hepatic tumor models of mice were established to initially explore the retention of HPTTG at the tumor site. Cy5.5-HPTTG-6.8 was injected into the tail vein of the mice. The accumulation of Cy5.5-HPTTG-6.8 in mice was observed at 4, 8, 12, 24, 48, and 72 h after tail vein injection. The result is shown in Figure 2A, in 4 h postinjection, HPTTG had accumulated in the liver. The fluorescence image of tissues 72 h postinjection showed that Cy5.5-HPTTG-6.8 accumulated in the liver (Figure 2B,D), especially accumulated in the hepatic tumor site. It is shown that Cy5.5-HPTTG-6.8 can target to the tumor tissue. We also found that the kidneys had fluorescent signals too; Cy5.5 being a small molecule may have separated from Cy5.5-HPTTG-6.8 and was metabolized by the urinary system. HPTTG accumulated in the liver may be due to the mononuclear phagocyte system (MPS). The liver and spleen are major biological barriers to transporting substances because they can sequester the majority of substances and prevent delivery to diseased tissue.\textsuperscript{44} Therefore, HPTTG would be trapped by the liver due to the MPS, and it would target to the tumor due to the tumor acidic microenvironment.

In order to rule out the impact of MPS on the accumulation of HPTTG, the subcutaneous tumor model of mice was used for embolization experiments. The accumulation of Cy5.5-HPTTG-6.8 in the tumor site was observed after 4, 8, 12, 24, 48, and 72 h postinjection. As shown in Figure 2A, after 4 h, Cy5.5-HPTTG-6.8 accumulated in the tumor site, but there was a large amount of Cy5.5-HPTTG-6.8 accumulated in the liver. After 72 h, Cy5.5-HPTTG-6.8 accumulated in the tumor site; there was no accumulation in the liver. This phenomenon may due to the MPS; a large number of the injected substances are cleared from the bloodstream by cells of the MPS. In vivo, MPS is a part of the immune system, which is built by immune and architectural cells and is located in organs such as the liver and spleen. The MPS can remove foreign materials from the bloodstream. Some substances can pass through the MPS and reach to the tumor site, but because of the MPS, the substances’ velocity reduces 1000-fold as they enter and traverse the liver.\textsuperscript{45} When HPTTG enters and traverses to the liver, it is retained by the liver because of MPS. Given time, HPTTG can pass through the MPS and circulate in the body. When HPTTG reaches the tumor site, it converts to hydrogel because of the acidic microenvironment and remains at the tumor site; this may be due to the strong hydrophilicity of HPTTG, and it may help it to pass through the MPS.

Moreover, an increment in the vascular permeability and tumor accumulation is termed as the enhanced permeability and retention (EPR) effect, which is a main mechanism for passive tumor targeting.\textsuperscript{46} The EPR effect is a unique phenomenon of solid tumors. While a large number of substances exploit the EPR effect for tumor uptake, the EPR effect is a molecular-weight-dependent phenomenon.\textsuperscript{47,48} For the EPR effect, the size of the macromolecule is a crucial factor with respect to uptake by the tumor; this is observed for macromolecules with molecular weights greater than 20 kDa.\textsuperscript{49} The accumulation of HPTTG at the tumor site may be caused by the EPR effect instead of being pH-responsive. To investigate whether the accumulation of the HPTTG was indeed due to the tumor acidic microenvironment response rather than the EPR effect, subcutaneous tumor models of mice were used. One group with Cy5.5-HPTTG-6.8 (30 mg/kg) was injected through the tail vein; another group was injected Cy5.5-HPTTG-3.5 through the tail vein as a control. The accumulation of the HPTTG in the tumor site of the mice was observed at 4, 8, 12, 24, 48, and 72 h postinjection, separately. As shown in Figure 2A, after 72 h, Cy5.5-HPTTG-6.8 accumulated in the tumor site. However, the Cy5.5-HPTTG-3.5 did not accumulate in the tumor site via blood circulation; instead, it accumulated in the liver. As is shown in Figure 2A,B,E, Cy5.5-HPTTG-6.8 accumulated at the tumor site, but Cy5.5-HPTTG-3.5 did not accumulate. This proves that the accumulation of HPTTG is mainly due to the tumor acidic microenvironment rather than the EPR effect.

Intercellular hypertension, hypoxia, and acidosis are the characteristics of the tumor microenvironment.\textsuperscript{50} Solid pressure generated by proliferating cancer cells further impairs the blood flow, leading to interstitial hypertension. Intersitial hypertension potentially hampers accumulation of macromolecules at the tumor site.\textsuperscript{51} In order to prove that HPTTG has the ability to accumulate in peripheral blood vessels of the tumors, FITC-HPTTG-6.8 was injected into the tail vein of the mice. After 24 h, we explored the fluorescence distribution at the tumor site via fluorescence microscopy. As is shown in Figure 2C, FITC-HPTTG-6.8 accumulated and distributed through the entire tumor. This indicates that HPTTG can accumulate in all peripheral blood vessels of the tumor site. In addition, it is found that the fluorescent signal of FITC-HPTTG-6.8 is weakened from the outside to the inside, proving that interstitial hypertension potentially hampers accumulation of FITC-HPTTG-6.8; however, this hampering is limited.

To further explore the embolization effect of HPTTG at the tumor site, the VX2 tumor-bearing rabbits were used. Although the VX2 tumors in rabbits are not of hepatic origin, the VX2 tumor is commonly used to model liver tumor embolization, because the VX2 tumor grows rapidly and has a similarity in blood supply to human HCC.\textsuperscript{10} The accumulation of the HPTTG in rabbits was observed using single photon emission computed tomography (SPECT). SPECT is one of the most important nuclear medicine imaging techniques in nuclear medicine and has made significant contributions in the imaging and therapeutic fields of cancer and cardiovascular diseases. Compared to fluorescence imaging in vivo, SPECT can analyze quantitatively and has a deep penetration capability. The nuclides commonly used in SPECT are mainly 131I, 18F, 99mTc, and 111In.\textsuperscript{52–54} 131I can not only be used to image tumor sites but also have therapeutic effects on tumor sites.\textsuperscript{55} It also has a longer half-life ($t_{1/2} = 8.01$ days), which can overcome the shortcomings of radionuclides such as 18F whose half-life in the human body is too short. To explore the feasibility of 131I-HPTTG for in vivo tumor SPECT imaging and the targeting of hepatic tumors, as is shown in Figure 2G, 131I-HPTTG accumulated at the tumor. Moreover, the rabbits’ thyroid gland and bladder had strong radioactivity. This phenomenon was probably due to the presence of some unmarked 131I molecules on HPTTG. Free 131I can be absorbed by the thyroid gland and eventually become metabolized by the urinary system.

The radiopacity and vascular embolization ability of HPTTG were assessed under digital subtraction angiography (DSA). Iohexol was selected to examine the feasibility of HPTTG as an embolic agent in occluding the blood supply artery of the tumor. The angiographic images of the embolized hepatic tumor of the rabbits showed that the embolic agent in occluding the blood supply artery of the tumor. The purpose of embolization is to reduce the blood supply around the tumor site, cut off the nutrient supply of the tumor, and finally inhibit its growth.\textsuperscript{43} We first examined whether HPTTG could accumulate at the tumor site, exploring its rate of accumulation at the tumor site. Biological studies were conducted in animal models. In this study, hepatic tumor models of mice were established to initially explore the retention of HPTTG at the tumor site. Cy5.5-HPTTG-6.8 was injected into the tail vein of the mice. The accumulation of Cy5.5-HPTTG-6.8 in mice was observed at 4, 8, 12, 24, 48, and 72 h after tail vein injection. The result is shown in Figure 2A, in 4 h postinjection, HPTTG had accumulated in the liver. The fluorescence image of tissues 72 h postinjection showed that Cy5.5-HPTTG-6.8 accumulated in the liver (Figure 2B,D), especially accumulated in the hepatic tumor site. It is shown that Cy5.5-HPTTG-6.8 can target to the tumor tissue. We also found that the kidneys had fluorescent signals too; Cy5.5 being a small molecule may have separated from Cy5.5-HPTTG-6.8 and was metabolized by the urinary system. HPTTG accumulated in the liver may be due to the mononuclear phagocyte system (MPS). The liver and spleen are major biological barriers to transporting substances because they can sequester the majority of substances and prevent delivery to diseased tissue.\textsuperscript{44} Therefore, HPTTG would be trapped by the liver due to the MPS, and it would target to the tumor due to the tumor acidic microenvironment.

In order to rule out the impact of MPS on the accumulation of HPTTG, the subcutaneous tumor model of mice was used for embolization experiments. The accumulation of Cy5.5-HPTTG-6.8 in the tumor site was observed after 4, 8, 12, 24, 48, and 72 h postinjection. As shown in Figure 2A, after 4 h, Cy5.5-HPTTG-6.8 accumulated in the tumor site, but there was a large amount of Cy5.5-HPTTG-6.8 accumulated in the liver. After 72 h, Cy5.5-HPTTG-6.8 accumulated in the tumor site; there was no accumulation in the liver. This phenomenon may due to the MPS; a large number of the injected substances are cleared from the bloodstream by cells of the MPS. In vivo, MPS is a part of the immune system, which is built by immune and architectural cells and is located in organs such as the liver and spleen. The MPS can remove foreign materials from the bloodstream. Some substances can pass through the MPS and reach to the tumor site, but because of the MPS, the substances’ velocity reduces 1000-fold as they enter and traverse the liver.\textsuperscript{45} When HPTTG enters and traverses to the liver, it is retained by the liver because of MPS. Given time, HPTTG can pass through the MPS and circulate in the body. When HPTTG reaches the tumor site, it converts to hydrogel because of the acidic microenvironment and remains at the tumor site; this may be due to the strong hydrophilicity of HPTTG, and it may help it to pass through the MPS.

Moreover, an increment in the vascular permeability and tumor accumulation is termed as the enhanced permeability and retention (EPR) effect, which is a main mechanism for passive tumor targeting.\textsuperscript{46} The EPR effect is a unique phenomenon of solid tumors. While a large number of substances exploit the EPR effect for tumor uptake, the EPR effect is a molecular-weight-dependent phenomenon.\textsuperscript{47,48} For the EPR effect, the size of the macromolecule is a crucial factor with respect to uptake by the tumor; this is observed for macromolecules with molecular weights greater than 20 kDa.\textsuperscript{49} The accumulation of HPTTG at the tumor site may be caused by the EPR effect instead of being pH-responsive. To investigate whether the accumulation of the HPTTG was indeed due to the tumor acidic microenvironment

https://dx.doi.org/10.1021/acscentsci.0c00506
ACS Cent. Sci. 2020, 6, 1977−1986
embolization. Before embolization, the hepatic tumor could be seen clearly after injecting a contrast agent into the liver. Also, the blood supply artery of the tumor and its peripheral branches can be observed clearly. After 12 h of embolization, iohexol was injected into the hepatic artery through a 4F microcatheter, and the hepatic tumor was invisible on the radiography image (Figure 2H). This phenomenon can be attributed to the obstruction of blood flow into the hepatic tumor due to the embolization of HPTTG, demonstrating the tumor being successfully occluded by using HPTTG.

The result shown in Table S1 explores the median lethal dose of HPTTG. When the concentration of HPTTG reached 200 mg/kg, more than half of the mice died, indicating that the median lethal concentration of HPTTG is 200 mg/kg.

To evaluate embolization effects, subcutaneous tumor-bearing mice were used. HPTTG was injected through the tail vein of the subcutaneous tumor mice. The therapeutic effect of HPTTG was observed on the tumor model. One group with HPTTG-6.8 (150 mg/kg) was injected through the tail vein, and one group with HPTTG-3.5 was injected through the tail vein; another group acts as a blank control. After 20 days of vascular embolization, tumors in the treatment group gradually shrunk in 20 days (Figure 3A–C). Compared with HPTTG-6.8, HPTTG-3.5 shows almost no treatment effect. The mouse body weight increased steadily (Figure 3D). Such results show that embolization is very effective for the tumor. In addition, after 60 days, the survival rate of mice is maintained at about 80% (Figure 3E). In summary, The HPTTG can be used for noninvasive embolization of tumors.

We made a pathological analysis of the tumor, as is shown in Figure 3F. In the experiment, hematoxylin–eosin staining (H&E staining), Ki-67 staining, and CD31 staining were used for pathological analysis. H&E staining is the best-known and is the most commonly used histological overview staining technique in pathology to study pathological changes in tissue samples. Ki-67 is one of the most widely used proliferation-
associated markers, and it is one of the methods of assessing the proliferation and growth of tumors' immunohistochemical staining.\textsuperscript{57} CD31 is one of the most widely used vascular markers and is used to assess tumor angiogenesis.\textsuperscript{58} Comparing the administered group with the control group, a large number of active tumors continue to exist in the control group, while the tumor tissues in the administered group undergo necrosis. Comparing the pathological analysis of the main organs of the heart, spleen, and kidney of other mice after embolization treatment, there are no significant histopathological changes (Figure 3G). The above results demonstrate that the substance can be applied to an antitumor treatment of an organism as an embolization agent for treating tumors.

To evaluate the embolic performance of tumor-bearing rabbits models, HPTTG was injected into the ear vein of the VX2 tumor-bearing rabbits, and the therapeutic effect of the substance on the rabbit liver cancer was observed. Tumor sizes were detected by MRI at 0, 5, 10, 15, and 20 days. As is shown in Figure 4A,B, after embolization treatment with HPTTG, the tumor gradually shrank in 20 days. Compared with the control group, the rabbits' body weight remained relatively stable in 20 days (Figure 4C). These results show that HPTTG has an excellent embolic performance for the tumor. In addition, after 60 days, the survival rate of rabbits is maintained over 70% (Figure 4D).

Figure 4E is a pathological analysis of rabbit VX2 hepatic tumors after 20 days of treatment. As is shown in Figure 4E, there is a large amount of tissue necrosis at the tumor site of the treat group, which did not affect normal liver tissue. By comparing the pathological analysis of the main organs such as the heart, spleen, and kidney of the rabbit after embolization treatment (Figure 4F), there was no significant histopathological change. The results demonstrate that HPTTG can be used in the treatment of tumors.

3. CONCLUSION

In this study, the new method of noninvasive and target embolization therapy by intravenous injection was developed and successfully achieved based on a sort of acidic microenvironment-responsive hyperbranched poly(amino acid) (HPTTG). The hyperbranched poly(amino acid)s, poly[l-threonine-l-(l-glutamic acid-ran-l-tyrosine)]s (HPTTG), were synthesized via ring-opening polymerization, and the pH value of sol-to-gel phase transition with decreasing pH was controlled.
at 3.5–10.0 by adjusting the ratio of L-glutamic acid and L-tyrosine in copolymers. HPTTG have excellent biodegradability and biocompatibility. In particular, HPTTG-6.8 (the pH value of sol-to-gel phase transition is 6.8) showed excellent target and embolization therapy effects. H22 tumor-bearing mice (subcutaneous and hepatic tumor models) and VX2 tumor-bearing rabbits (hepatic tumor models) were used as animal models after intravenous injection; the targeting accumulation, vascular embolization, and therapeutic effect were evaluated by in vivo fluorescence images, single photon emission computed tomography (SPECT), digital subtraction angiography (DSA), and magnetic resonance imaging (MRI), etc. The results indicate that HPTTG-6.8 accumulates the most at the tumor site in 8 h into postinjection; blood vessels of the tumors were occluded, and the tumors were inhibited and necrotized of all mice and rabbits in roughly 20 days. The survival rates of mice were maintained at more than 80% in 60 days. The survival rates of rabbits were maintained at more than 70% in 60 days. These novel embolic materials and method show great potential. Not only can they be used for noninvasive target-embolization therapy of many solid tumors, they can also be used as a multifunctional platform for combined theranostics such as a combination with controlled release, thermal ablation, multimodal imaging, synergistic therapy, etc.

**ASSOCIATED CONTENT**

- Supporting Information
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c00506.
  Details of experimental and supplementary experimental characterization, 1H NMR and GPC (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Dedai Lu — Key Laboratory of Eco-Functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-Environmental Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China; orcid.org/0000-0002-4161-5373; Email: ludedai@126.com, ludedai@nwnu.edu.cn

**Authors**

Jiachen Wang — Key Laboratory of Eco-Functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-Environmental Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China

Yunfei Li — Key Laboratory of Eco-Functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-Environmental Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China

Yongyong Zhang — Key Laboratory of Eco-Functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-Environmental Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China

Lili Yu — Key Laboratory of Eco-Functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-Environmental Polymer Materials of Gansu Province, College of Chemistry and Chemical Engineering, Northwest Normal University, Lanzhou 730070, China

**Author Contributions**

Dedai Lu designed and supervised the whole work and wrote the article with Jiachen Wang. Jiachen Wang synthesized most of the copolymers, participated in most of the relevant experiment work, and wrote the article with Dedai Lu. Yunfei Li participated in the copolymers synthetic work and animal experiments. Yongyong Zhang participated in the synthesis of monomers and characterization of copolymers. Lili Yu and Tingting Xu participated in animal experiments; they also participated partly in the medical images tests. Gaojun Teng has given a lot of guidance and assisted in animal experiment design and result analysis. Ziqiang Lei has given advice and assisted in experimental designing and article writing work.

**Funding**

This work was supported in part by the National Natural Science Foundation of China (21865029), West Light Foundation of the Chinese Academy of Sciences (2018[99]), Support Program for Longyuan Youth and Fundamental Research Funds for the Universities of Gansu Province ((2017)121), and Innovation Team Project of NWNU(NWNU-LKQN-16-2). We also thank Key Laboratory of Eco-functional Polymer Materials of the Ministry of Education, Key Laboratory of Eco-environmental Polymer Materials of Gansu Province, for financial support.

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS**

HCC, hepatocellular carcinoma; TAE, transcatheter arterial embolization; L-Thr-NCA, L-threonine-N-carboxyanhydride; Cbz-L-Tyr-NCA, benzyl chloroformate protected L-tyrosine-N-carboxyanhydride; BL-Glu-NCA, L-glutamic acid-S-benzylester-N-carboxyanhydride; HPTTG, hyperbranched poly-[Thr-(Tyr-ran-Glu)]; PT, calculated prothrombin time; APTT, activated partial thromboplastin time; SPECT, single photon imaging.
emission computed tomography; DSA, digital subtraction angiography

REFERENCES

(1) Wang, Q.; Qian, K.; Liu, S.; Yang, Y.; Liang, B.; Zheng, C.; Yang, X.; Xu, H.; Shen, A. Q. X-ray Visible and Uniform Alginic Microspheres Loaded with in Situ Synthesized BaSO4 Nanoparticles for in Vivo Transcatheter Arterial Embolization. Biomacromolecules 2015, 16, 1240−1246.
(2) Chiang, P.-F.; Peng, C.-L.; Shih, Y.-H.; Cho, Y.-H.; Yu, C.-S.; Kuo, Y.-M.; Shieh, M.-J.; Luo, T.-Y. Biodegradable and Multifunctional Microspheres for Treatment of Hepatoma through Transarterial Embolization. ACS Biomater. Sci. Eng. 2018, 4, 3425−3433.
(3) Zhou, H.; Kong, M.; Cheng, X.; Li, J.; Li, J.; Chen, X. Investigation of acetylated chitosan microspheres as potential chemoembolic agents. Colloids Surf., B 2014, 123, 387−394.
(4) Wang, Y.-X.; Zhu, X.-M.; Liang, Q.; Cheng, C. H. K.; Wang, W.; Leung, K. C.-F. In Vivo Chemomobilization and Magnetic Resonance Imaging of Liver Tumors by Using Iron Oxide Nanoshell/ Doxorubicin/Poly(vinyl alcohol) Hybrid Composites. Angew. Chem., Int. Ed. 2014, 53, 4812−4815.
(5) Li, Z.; Di, C.; Li, S.; Yang, X.; Nie, G. Smart Nanotheerapeutic Targeting of Tumor Vasculature. Acc. Chem. Res. 2019, 52, 2703−2712.
(6) Liu, Y.; Peng, X.; Qiao, K.; Ma, Y.; Wan, J.; Li, H.; Zhang, H.; Zhou, G.; Xiong, B.; Zhao, Y.; Zheng, C.; Yang, X. Temperature sensitive p(N-isopropylacrylamide-co-acrylic acid) modified gold nanoparticles for trans-arterial embolization and angiography. J. Mater. Chem. B 2017, 5, 907−916.
(7) Poursa, A.; Jensen, M. M.; Huo, E.; Ghandehari, H. Polymeric materials for embolic and chemoembolic applications. J. Controlled Release 2016, 240, 414−433.
(8) Zhou, X.; Kong, M.; Cheng, X. J.; Feng, C.; Li, J.; Li, J.; Chen, X. G. In vitro and in vivo evaluation of chitosan microspheres with different deacetylation degree as potential embolic agent. Carbohydr. Polym. 2014, 113, 304−313.
(9) Hagit, A.; Soenke, B.; Johannes, B.; Shlomo, M. Synthesis and Characterization of Dual Modality (CT/MRI) Core-Shell Microspheres for Embolization Purposes. Biomacromolecules 2010, 11, 1600−1607.
(10) Hu, J.; Albadhawi, H.; Chong, B. W.; Deipolay, A. R.; Sheth, R. A.; Khademhosseini, A.; Olku, R. Advances in Biomaterials and Technologies for Vasculization Embolization. Adv. Mater. 2019, 31, 1901071.
(11) Shen, L.; Zhang, Y.; Zhang, J.; Wang, T.; Li, H.; Wang, Y.; Quan, D. Reversed Lipid-Based Nanoparticles Dispersed in Iodized Oil for Transarterial Chemoembolization. ACS Appl. Mater. Interfaces 2019, 11, 20642−20648.
(12) Zhao, Y.; Zheng, C.; Wang, Q.; Fang, J.; Zhou, G.; Zhao, H.; Yang, Y.; Xu, H.; Feng, G.; Yang, X. Permanent and Peripheral Embolization: Temperature-Sensitive p(N-isopropylacrylamide-cobutyl Methylacrylate) Nanogel as a Novel Blood-Vessel-Embolic Material in the Interventional Therapy of Liver Tumors. Adv. Funct. Mater. 2011, 21, 2035−2042.
(13) Shi, X.; Gao, H.; Dai, F.; Feng, X.; Liu, W. A thermoresponsive superamolecular copolymer hydrogel for the embolization of kidney arteries. Biomater. Sci. 2016, 4, 1673−1681.
(14) Liu, Y.; Peng, X.; Qian, K.; Ma, Y.; Wan, J.; Li, H.; Zhang, H.; Zhou, G.; Xiong, B.; Zhao, Y.; Zheng, C.; Yang, X. Temperature sensitive p(N-isopropylacrylamide-co-acrylic acid) modified gold nanoparticles for trans-arterial embolization and angiography. J. Mater. Chem. B 2017, 5, 907−916.
(15) Zhou, C.; Yao, Q.; Zhang, H.; Guo, X.; Liu, J.; Shi, Q.; Huang, S.; Xiong, B. Combining transcatheter arterial embolization with iodized oil containing Apatinib inhibits HCC growth and metastasis. Sci. Rep. 2020, 10, 2964.
(16) Nguyen, Q. V.; Lee, M.-S.; Lym, J. S.; Kim, Y. I.; Lee, D. S. A novel sulfamethazine-based pH-sensitive copolymer for injectable radiopaque embolic hydrogels with potential application in hepatocellular carcinoma therapy. Polym. Chem. 2016, 7, 5805−5818.
(17) Nguyen, J. S.; Nguyen, Q. V.; Ahn, D. W.; Huyhn, C. T.; Jae, H. J.; Kim, Y. I.; Lee, D. S. A novel sulfamethazine-based pH-sensitive hydrogel with potential application for transcatheter arterial chemoembolization therapy. Acta Biomater. 2016, 41, 253−263.
(18) Lee, B. H.; West, B.; McMlore, R.; Pauken, C.; Vernon, B. L. In Situ Injectable Physically and Chemically Gelling NIPAAm-Based Copolymer System for Embolization. Biomacromolecules 2006, 7, 2059−2064.
(19) Zhu, A. X.; Salem, R. Combining Transarterial Chemoembolization With Radiofrequency Ablation for Hepatocellular Carcinoma: One Step Forward. J. Clin. Oncol. 2013, 31, 406−408.
(20) Ashour, R.; Aziz-Sultan, A. Preoperative Tumor Embolization. Neurosurgery Clinics of North America 2014, 25, 607−617.
(21) Masoud, H.; Nguyen, T.; Norbass, A. S. Tumor Embolization. Neurocritical Care Management of the Neurosurgical Patient 2018, 391−400.
(22) Boston, S. Transarterial Embolization and Chemoembolization. Veterinary Image-Guided Interventions 2015, 629−634.
(23) Wang, L.; HUO, M.; Chen, Y.; Shi, J. Tumor Microenvironment-Enabled Nanotherapy. Adv. Healthcare Mater. 2018, 7, 1701156.
(24) Dai, X.; Xu, C.; Sun, X.; Chen, X. Nanoparticle design strategies for enhanced anticancer therapy by exploiting the tumor microenvironment. Chem. Soc. Rev. 2017, 46, 3830−3852.
(25) Guo, X.; Wang, L.; Wei, X.; Zhou, S. Polymer-based drug delivery systems for cancer treatment. J. Polym. Sci., Part A: Polym. Chem. 2016, 54, 3525−3550.
(26) Corbet, C.; Feron, O. Tumour Acidosis: From the Passenger to the Driver’s Seat. Nat. Rev. Cancer 2017, 17, 577−593.
(27) Prasad, P.; Gordjio, C. R.; Abbasi, A. Z.; Maeda, A.; Ip, A.; Rauth, A. M.; DaCosta, R. S.; Wu, X. Y. Multifunctional Albumin MnO2 Nanoparticles Modulate Solid Tumor Microenvironment by Attenuating Hypoxia, Acidosis, Vascular Endothelial Growth Factor and Enhance Radiation Response. ACS Nano 2014, 8, 3202−3212.
(28) Tseng, S.-J.; Kempson, I. M.; Huang, K.-Y.; Li, H.-J.; Fa, Y.-C.; Ho, Y.-C.; Liao, Z.-X.; Yang, P.-C. Targeting Tumor Microenvironment by Bioreduction-Activated Nanoparticles for Light-Triggered Virotherapy. ACS Nano 2018, 12, 9994−9902.
(29) Byrne, M.; Murphy, R.; Kapetanakis, A.; Ramsey, J.; Cryan, S.-A.; Heise, A. Star-Shaped Polypeptides: Synthesis and Opportunities for Delivery of Therapeutics. Macromol. Rapid Commun. 2015, 36, 1862−1876.
(30) Mintzer, M. A.; Grinstaff, M. W. Biomedical applications of dendrimers: a tutorial. Chem. Soc. Rev. 2011, 40, 173−190.
(31) Kasza, G.; Kali, G.; Domjan, L.; Petö, L.; Szarka, G.; Ivan, B. Synthesis of Well-Defined Pthalimide Monofunctional Hyperbranched Polyglycerols and Its Transformation to Various Conjugation Relevant Functionalities. Macromolecules 2017, 50, 3078−3088.
(32) Wang, D.; Zhao, T.; Zhu, X.; Yan, D.; Wang, W. Bioapplications of hyperbranched polymers. Chem. Soc. Rev. 2015, 44, 4023−4071.
(33) Lu, D.; Li, Y.; Wang, X.; Li, T.; Zhang, Y.; Guo, H.; Sun, S.; Wang, X.; Zhang, Y.; Lei, Z. All-in-one hyperbranched polypeptides for surgical adhesives and interventional embolization of tumors. J. Mater. Chem. B 2016, 8, 7511−7520.
(34) Alexius, F.; Prigden, E.; Molnar, L. K.; Farokhzad, O. C. Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles. Mol. Pharmaceutics 2008, 5, 505−515.
(35) Dai, Y.; Xu, C.; Sun, X.; Chen, X. Nanoparticle design strategies for enhanced anticancer therapy by exploiting the tumor microenvironment. Chem. Soc. Rev. 2017, 46, 3830−3852.
(38) Sadat, S. M. A.; Jahan, S. T.; Haddadi, A. Effects of Size and Surface Charge of Polymeric Nanoparticles on in Vitro and in Vivo Applications. *J. Biomater. Nanobiotechnol.* 2016, *7*, 91–108.

(39) He, C.; Hu, Y.; Yin, L.; Tang, C.; Yin, C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* 2010, *31*, 3657–3666.

(40) Lu, D.; Zhang, Y.; Li, Y.; Luo, C.; Wang, X.; Guan, X.; Ma, H.; Zhao, X.; Wei, Q.; Lei, Z. Preparation and properties of reversible hydrogels based on triblock poly(αmino acid)s with tunable pH-responsivity across a broad range. *J. Polym. Sci., Part A: Polym. Chem.* 2017, *55*, 207–212.

(41) Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Res.* 1986, *46*, 6387.

(42) Lale, S. V.; Aswathy, R. G.; Aravind, A.; Kumar, D. S.; Koul, V. AS1411 Aptamer and Folic Acid Functionalized pH-Responsive ATRP Fabricated pPEGMA–PCL–pPEGMA Polymeric Nanoparticles for Targeted Drug Delivery in Cancer Therapy. *Biomacromolecules* 2014, *15*, 1737–1752.

(43) Krysl, J.; Kumpe, D. A. Embolization agents: A review. *Technol. Vasc. Interv. Radiol.* 2000, *3*, 158.

(44) Vaidya, S.; Tozer, K. R.; Chen, J. An overview of embolic agents. *Semin. Intervent. Radiol.* 2008, *25*, 204.

(45) Tsoi, K. M.; MacParland, S. A; Ma, X.-Z.; Spetzler, V. N. Mechanism of hard-nanomaterial clearance by the liver. *Nat. Mater.* 2016, *15*, 1212–1221.

(46) Lee, S. Y.; Ko, S.-H.; Shim, J.-S.; Cho, H.-J. Correction to “Tumor Targeting and Lipid Rafts Disrupting Hyaluronic Acid-Cyclodextrin-Based Nanoassembled Structure for Cancer Therapy. *ACS Appl. Mater. Interfaces* 2018, *10*, 36628–36640.

(47) Wilhelm, S.; Tavares, A. J.; Dai, Q.; Ohta, S.; Audet, J.; Dvorak, H. F.; Chan, W. C. W. Analysis of nanoparticle delivery to tumours. *Nat. Rev. Mater.* 2016, *1*, 16014.

(48) Mouli, S. K.; Tyler, P.; McDevitt, J. L.; Eifler, A. C. Image-Guided Local Delivery Strategies Enhance Therapeutic Nanoparticle Uptake in Solid Tumors. *ACS Nano* 2013, *7*, 7724–7733.

(49) Haag, R.; Kratz, F. Polymer Therapeutics: Concepts and Applications. *Angew. Chem., Int. Ed.* 2006, *45*, 1198–1215.

(50) Jain, R. K. Normalization of Tumor Vasculature: An Emerging Concept in Antiangiogenic Therapy. *Science* 2005, *307*, 58–62.

(51) Danquah, M. K.; Zhang, X. A.; Mahato, R. I. Extravasation of polymeric nanomedicines across tumor vasculature. *Adv. Drug Delivery Rev.* 2011, *63*, 623–639.

(52) Jain, R. K.; Stylianopoulos, T. Delivering nanomedicine to solid tumors. *Nat. Rev. Clin. Oncol.* 2010, *7*, 653–664.

(53) Liu, Z.; Huang, J.; Dong, C.; Cui, L. 99mTc-Labeled RGD-BBN Peptide for Small-Animal SPECT/CT of Lung Carcinoma. *Mol. Pharmaceutics* 2012, *9*, 1409–1417.

(54) Wen, X.; Shi, C.; Xu, D.; Zhang, P. Radioiodinated Portable Albumin Binder as a Versatile Agent for in vivo Imaging with Single-Photon Emission Computed Tomography. *Mol. Pharmacaceutics* 2019, *16*, 816–824.

(55) Cao, J.; Wei, Y.; Zhang, Y.; Wang, G. Iodine-rich polymersomes enable versatile SPECT/CT imaging and potent radiotracer therapy for tumor in vivo. *ACS Appl. Mater. Interfaces* 2019, *11*, 18953–18959.

(56) Schrödel, A. Übersichtsfarbungen mit Hamatoxylin und Eosin (H&E). *Biol. Unserer Zeit* 2012, *42*, 153–153.

(57) Wang, B.; Weng, S.-L.; Chau, G.-Y.; Tsay, S.-H.; Chi, C.-W.; Lee, T.-G.; Wu, L.-H.; Wu, C.-W.; Lui, W.-Y. Ki-67 expression as a prognostic marker in patients with hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 1998, *13*, 273–279.

(58) Chen, B.; Gao, A.; Tu, B.; Wang, Y.; Yu, X.; Wang, Y.; Xiu, Y.; Wang, B.; Fan, Y.; Huang, Y. Metabolic modulation via mTOR pathway and anti-angiogenesis remodels tumor microenvironment using PD-L1-targeting codelivery. *Biomaterials* 2020, *255*, 120187.