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BRIEF ARTICLE

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Synthesis and Biological Evaluation of a Polyglutamic Acid−Dopamine Conjugate: A New Antiangiogenic Agent

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ABSTRACT: Dopamine has previously been shown to inhibit angiogenesis in vitro and in vivo, but its clinical applications in this context are severely limited by its short half-life. Here we report the synthesis of a polyglutamic acid−dopamine conjugate and show that conjugation significantly extends (from 1 to 24 h) dopamine's antiangiogenic activity in vitro and in vivo. These findings form the basis for the development of a new class of agents for the treatment of angiogenesis-dependent diseases.

INTRODUCTION

The formation of new blood vessels (angiogenesis) is a process normally occurring in various physiological circumstances, for instance, during repair of tissue damage.[1] In a parallel manner, inappropriate activation or deactivation of angiogenesis often accompanies the onset of diseases, including cancer.[1,2] Many studies have reported that activation of angiogenesis in tumors (the so-called "angiogenic switch") transforms small, dormant cancers into invasive, metastatic forms.[3] As a logical consequence, antiangiogenic agents have been suggested as possible anticancer drugs, and some have now reached the marketplace (e.g., bevacizumab).[2,4] While such agents are normally considered non-toxic, they in fact display some severe side effects due to their poor specificity. For instance, inhibition of angiogenesis in tissues other than the target site is reported to interfere with general and postsurgery wound healing.[5]

Dopamine is a catecholamine neurotransmitter that mediates vital functions such as voluntary movement, behavior, memory, and reward.[6,7] Recent studies have shown that dopamine has a pivotal role in the regulation of angiogenesis and that this neurotransmitter is able to inhibit angiogenesis at non-toxic concentrations, with potential for the treatment of angiogenesis-dependent diseases.[8,9] Practically, however, such applications are severely hindered by the short duration of dopamine action in vivo (half-life typically 2 min).[10] This limitation is particularly significant in the context of antiangiogenic therapy, where steady blood levels of angiogenesis inhibitors are required to counterbalance the continuous stimulation of proangiogenic factors on endothelial cells.[11] A long circulating form of dopamine would therefore improve its therapeutic response and potentially reduce the frequency of administration, resulting in better patient compliance.

It is well-known that conjugation of a low molecular weight drug to a polymeric carrier prolongs its half-life and increases circulation time.[12,13] This is, for example, the case for a paclitaxel−polyglutamic acid (PGA) conjugate (currently in phase III clinical trial[14]) whose half-life was found to be ∼10 times higher than that of unconjugated paclitaxel and that was still detectable in plasma after 3 weeks from administration of a 175 mg/m^2 dose.[12] In addition, we have previously shown that the performance of a potent but neurotoxic antiangiogenic agent TNP-470 could be markedly improved (prolonged activity and reduced neurotoxicity) by conjugation to an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer.[15]

In the present work, we hypothesize that the formation of a polymer−dopamine conjugate would prolong the antiangiogenic effect of dopamine, thus translating the interesting findings on dopamine's activity into viable clinical applications. Here the synthesis and characterization of a PGA−dopamine conjugate are reported and the ability of the conjugate to exert an antiangiogenic effect over a prolonged period of time is described. As dopamine has been shown to prevent vascular endothelial growth factor (VEGF) mediated angiogenesis,[8] the antiangiogenic properties of PGA−dopamine were determined in two VEGF-dependent systems: an in vitro test concerning endothelial cells migration (using the scratch assay[16]) and an in vivo assessment examining vascular permeability (using the Miles assay[17]).

RESULTS

Synthesis and Characterization of the PGA-Dopamine Conjugate (3). Prior to the synthesis of a polymer−dopamine conjugate, a suitable carrier had to be identified. PGA was considered ideal because of its proven clinical safety, biodegradability, and multivalency, with the last allowing a high drug loading.[12−14] The synthesis of PGA−dopamine conjugate **3** started from the conversion of PGA sodium salt **1** (30 kDa) to the free acid **2** using a proton exchange resin (Scheme 1). Formation of **2** was confirmed by IR spectroscopy (appearance of the strong peak at 1700 cm^{-1} related to the C═O stretching of the carboxylic acid). Then conjugation of dopamine was achieved by *N*,*N*′-diisopropylcarbodiimide (DIC) mediated coupling with the carboxylic acid residues. In this reaction, only 50% of the glutamate (Glu) monomers were activated to maintain at least 50% of pendent carboxylic acid, thus ensuring solubility of the conjugate in water. The identity of **3** was confirmed by ^{1}H and ^{13}C NMR spectroscopic analysis (Figure 1A and Figure 1B). The broadening of the peaks of dopamine in the ^{1}H NMR spectrum of the conjugate, compared to the sharp peaks of the ^{1}H NMR spectrum of the free molecule, is often found after conjugation of low molecular weight molecules to polymers (additional information in the Experimental Section). The total drug content was determined

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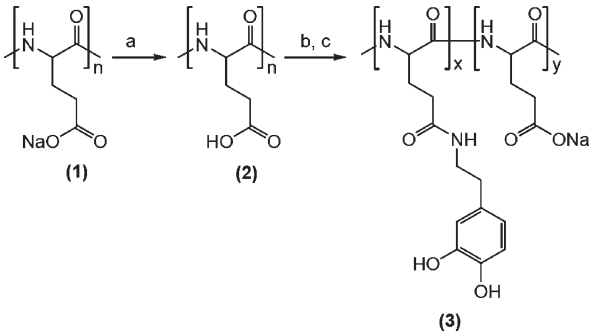
Scheme 1. Synthesis of PGA−Dopamine Conjugate (2)[a]



[a] Reagents and conditions: (a) Amberlite IR-120; (b) DIC, NHS, DMAP, DMF, dopamine, and DIEA; (c) $NaHCO_3$.

by ^{1}H NMR spectroscopy and was found to be typically 12% of conjugate weight with a good batch-to-batch reproducibility. This finding indicates that dopamine was attached to ∼14% of the Glu monomers (Table 1). In all polymer−drug conjugates, purification from the free drug was considered key, as this might contribute to the biological activity observed. Free dopamine was therefore removed by size exclusion chromatography, and residual dopamine after purification was always less than 1% w/w of total drug, as quantified by HPLC (Figure 1C, Table 1).

In Vitro Endothelial Cells Migration. Following synthesis of the PGA−dopamine conjugate, its ability to inhibit angiogenesis in vitro was assessed. The migration of endothelial cells is a key step in the formation of new blood vessels, and it is largely promoted by the presence of VEGF.[18] Therefore, the effect of PGA−dopamine on the migration of human umbilical vein endothelial cells (HUVEC) and its ability to revert VEGF-induced cell migration were probed in vitro using a scratch assay[16] (Figure 2). At a short incubation time (1 h) and at a concentration equivalent to a non-toxic dose of dopamine (1 μM, dopamine equiv),[8] PGA−dopamine significantly reduced HUVEC migration, confirming that this conjugate had antiangiogenic properties (Figure 2A). The extent of such inhibition was similar to that observed for free dopamine, at the same concentration and for the same incubation time (Figure 2A). Indeed, the VEGF stimulatory effect on cell migration was reduced to ∼50% in both cases (Figure 2A). At a longer incubation time (24 h), PGA−dopamine was still active (Figure 2B) and it was even more effective than at a shorter incubation time, with the VEGF stimulatory effect on HUVEC migration being completely inhibited. However, at 24 h, dopamine did not display any antiangiogenic effect (Figure 2B). This was not surprising given the short half-life of dopamine, and it was expected as dopamine's antiangiogenic action had previously been demonstrated only at short time frames (typically 1 h).[8] These results therefore illustrated that the PGA−dopamine conjugate was able to completely inhibit angiogenesis for at least 24 h, rendering it a far more effective antiangiogenic agent in vitro than dopamine itself.

In Vivo Vascular Permeability. To determine the viability of using the PGA−dopamine conjugate as a therapeutic, it was important to assess whether antiangiogenic activity would be retained in vivo. At the early stages of the angiogenic process, VEGF stimulates microvascular hyperpermeability, a condition that can be monitored and quantified in vivo using the Miles assay.[17,19,8] Within this assay, the effect of VEGF can be readily

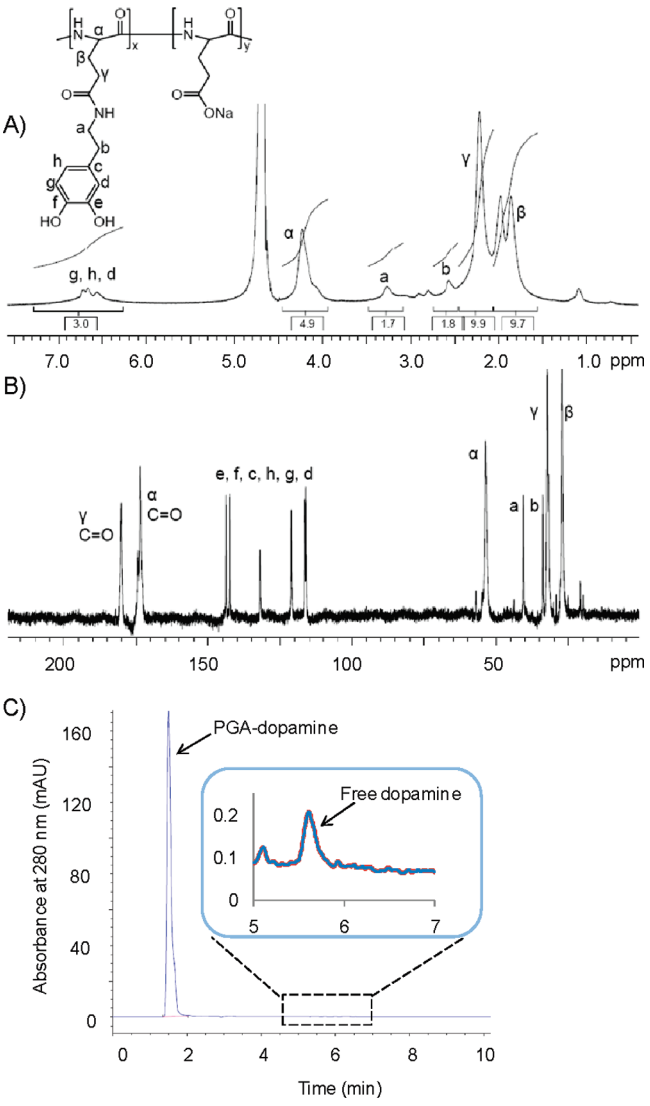


Figure 1. Characterization of PGA−dopamine: (a) ^{1}H NMR spectrum of PGA−dopamine; (b) ^{13}C NMR spectrum of PGA−dopamine; (c) HPLC chromatogram of PGA−dopamine (elution time, 1.5 min) and detection of the free dopamine (elution time, 5.6 min).

Table 1. Total and Free Dopamine Content in Different Batches of PGA−Dopamine Conjugates.

batch no.	total dopamine (% w/w)	conjugated monomers (% of total Glu)	free dopamine (% of total)
1	11.56	12.99	<1
2	10.79	11.67	<0.6
3	14.90	17.39	<0.6
4	10.84	12.06	<1

quantified by measuring the extravasation of Evans' blue dye at the sites of increased permeability. We assessed the ability of a single injection of PGA−dopamine (equivalent to a non-toxic 50 mg/kg dose of dopamine) to inhibit VEGF-induced vascular hyperpermeability in Balb/C female mice. The effect of a single injection of dopamine (also at 50 mg/kg) was also investigated as

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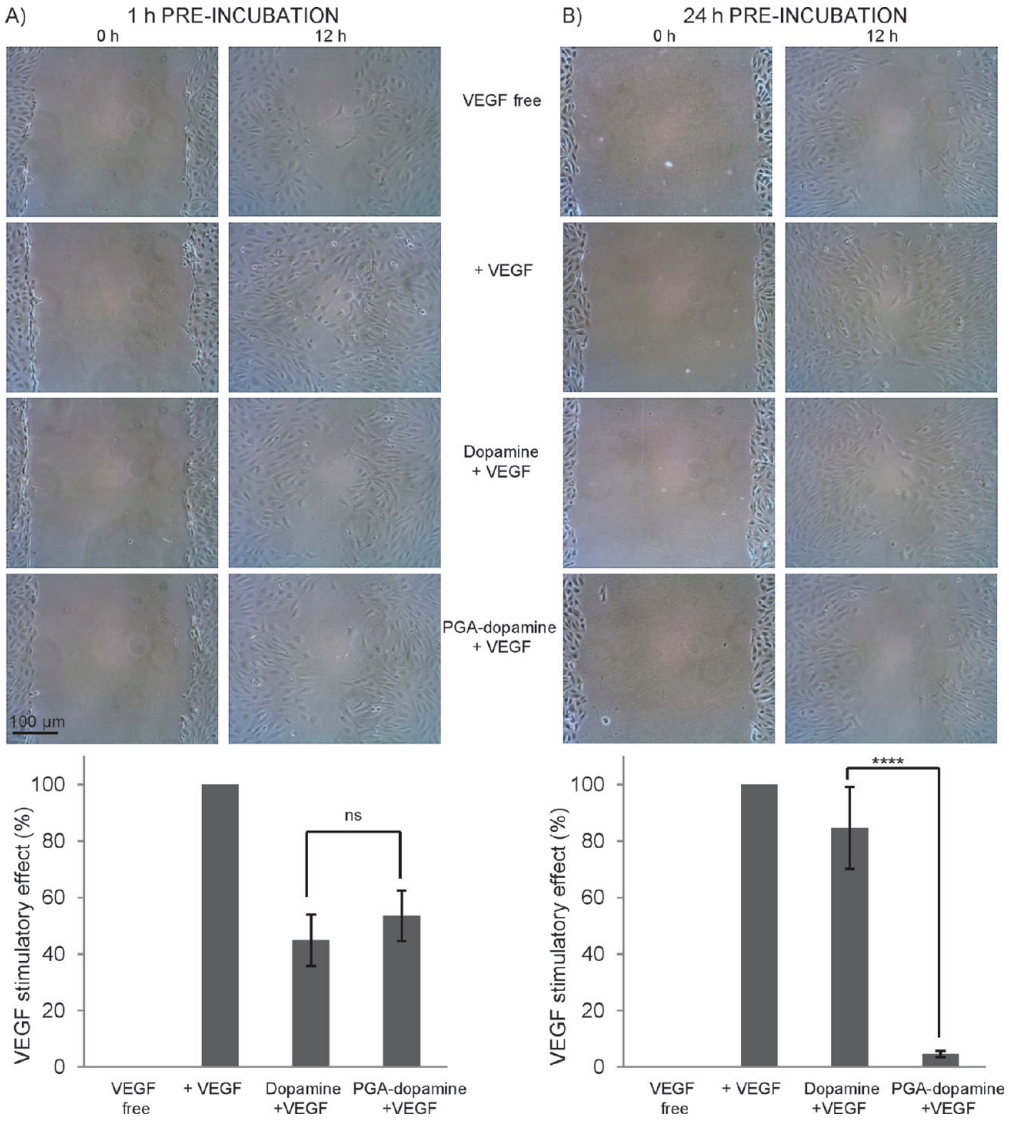

Figure 2. Effect of PGA−dopamine on the VEGF-induced HUVEC migration in vitro (scratch assay) after a short (A) or long (B) preincubation time with the treatments. Pictures were taken immediately after performing the scratch (0 h) and 12 h later. Bar charts represent the quantification of the inhibitory effect of PGA−dopamine, expressed as percentage of the scratched area covered by HUVEC after 12 h migration and normalized to presence/absence of VEGF (100% and 0% for VEGF and VEGF-free medium, respectively). Data are expressed as the mean ± standard error of the mean (SEM) (n = 6). Statistical significance was estimated by one-way ANOVA, followed by Bonferroni's post hoc test (ns = nonsignificant; ****, $p < 0.0001$).

control. In agreement with previous reports,[8] dopamine was able to inhibit vascular permeability for short time frames (dye extravasation reduced by ~60% at 1 h after dopamine injection), but this effect had disappeared completely at 24 h (Figure 3). In contrast, the PGA−dopamine conjugate displayed a much longer activity and was still active at 24 h (60% reduction of VEGF-induced vascular hyperpermeability) (Figure 3).

■ DISCUSSION

The development of effective angiogenesis modulators is of great interest in medicine, as changes in angiogenesis are often associated with the onset of human disease states. Basu et al.[8,9] reported the ability of dopamine to reduce angiogenesis, with potential for cancer treatment. These findings, while significant, are limited in practice by dopamine's short stability in vivo.[10] Dopamine is known to be subject to fast degradation (auto-oxidation)[20] and in vivo enzymatic metabolism, in the form of sulfoconjugation, oxidative deamination, and O-methylation.[10] All of the above are responsible for dopamine's short half-life. Our data show that by conjugating dopamine to PGA, its antiangiogenic activity is not only maintained but indeed prolonged in vitro and in vivo. This study suggests that PGA is able to protect dopamine from degradation, in line with previous reports describing the stabilizing effect of PGA conjugation on the fast degradation and loss of activity of common anticancer agents (e.g., camptothecin).[21]

In the wider context of angiogenesis-dependent diseases, in particular if one considers the use of antiangiogenic agents in cancer treatment, our findings have a more general impact. Polymer−drug conjugates carrying conventional anticancer agents have been extensively studied for cancer treatment, as they can passively accumulate into the tumor tissue, exploiting the well-described "enhanced permeability and retention (EPR) effect".[13] In the case of a PGA−dopamine conjugate, such localization would translate into

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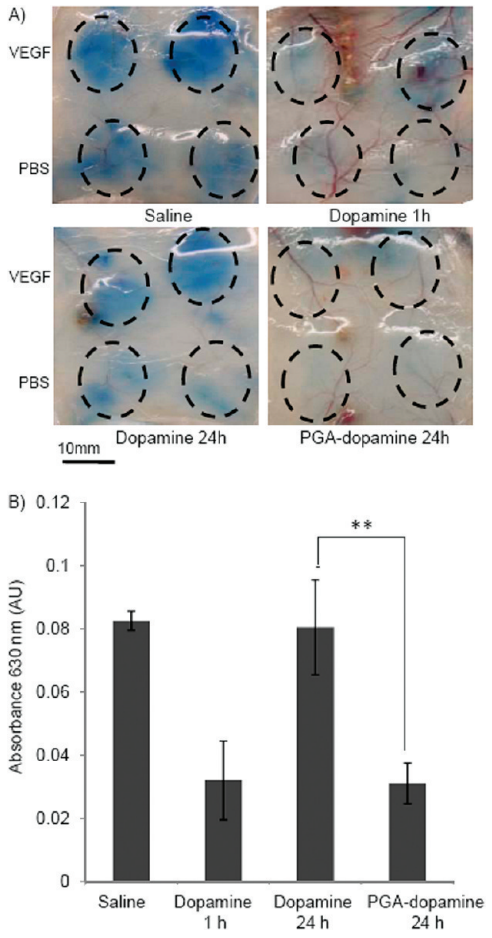

Figure 3. Effect of PGA−dopamine on the VEGF-induced microvascular hyperpermeability in vivo (Miles assay): (a) dye extravasation in mice treated with PGA-dopamine or free dopamine; (b) quantification of the extracted dye by UV measurement at 630 nm. Data are expressed as the mean ± standard error of the mean (SEM) (n = 4). Statistical significance was estimated by one-way ANOVA test, followed by Bonferroni's post hoc test (**, $p < 0.03$).

important therapeutic advantages. First, the inhibitory effect of dopamine on VEGF would be limited to the tumor site, where angiogenesis is pathological, and would not compromise the physiological activity of VEGF in healthy tissues.[22] This would constitute a significant advantage over current antiangiogenic treatments, which are largely nonspecific. Second, the possible side effects deriving from dopamine cardiac action would also be avoided.[23]

Unlike conventional small molecules, most polymer−drug conjugates, including the PGA−dopamine conjugate presented in this study, are intrinsically heterogeneous (a result of polymer polydispersity and of the coupling procedure). Therefore, particular attention needs to be given to scaling up methodology and batch to batch reproducibility during development. However, it is important to highlight that the production of similar types of systems has been successfully scaled up, allowing such agents to progress to the clinic[24] (e.g., PGA−paclitaxel[14]).

CONCLUSIONS

In this work, an effective method for the conjugation of dopamine to PGA has been established. We were also able to show that PGA−dopamine effectively inhibited angiogenesis in vitro and in vivo and, compared to the free dopamine, the conjugate had a significantly longer duration of activity (from 1 to 24 h). Further studies to clarify the mechanism of action and elucidate if dopamine release is essential for the conjugate's activity are currently under investigation. The ability of the conjugate to act as an anticancer agent and its potential to localize in the tumor tissue will also be explored.

EXPERIMENTAL SECTION

Synthesis and Characterization of PGA−Dopamine (3). A solution of PGA sodium salt (**1**) (0.50 g) (Alamanda Polymers, AL) in water (10 mL) was passed through a bed of Amberlite IR-120 resin (8 g) (Sigma-Aldrich, U.K.), collected, and freeze-dried to yield PGA-COOH (**2**) (0.38 g). PGA-COOH (**2**) (0.20 g, 1.5 mmol calculated as COOH) was flushed with N_2 and dissolved in dry DMF (10 mL) (Sigma-Aldrich, U.K.). DIC (0.09 g, 0.73 mmol) (Sigma-Aldrich, U.K.) was added, and the solution was stirred for 30 min before addition of NHS (0.19 g, 0.72 mmol) and DMAP (0.09 g, 0.74 mmol) (both from Sigma-Aldrich, U.K.). The reaction mixture was stirred overnight at room temperature. Dopamine (1.14 g, 6 mmol) and DIEA (0.76 g, 5.9 mmol) (Sigma-Aldrich, U.K.) were added in situ, and the solution was stirred for 48 h at room temperature before dialysis against water (membrane cut-off 3.5 kDa). The sodium salt of the PGA−dopamine conjugate was obtained by dissolving the product in the minimum amount of $NaHCO_3$ (0.5 M) (Sigma-Aldrich, U.K.). This solution was dialyzed against water and freeze-dried. PGA−DA was purified by size exclusion chromatography (Sephadex LH20, GE Healthcare, U.K.) using distilled water as eluent. PGA−dopamine was characterized by ^{1}H and ^{13}C NMR spectroscopic analysis (Bruker Avance III 700 MHz instrument, equipped with TOPSPIN 2.4 software). ^{1}H NMR (D_2O, δ ppm): 1.95 (2H, CH_2 β PGA), 2.29 (2H, CH_2 γ PGA), 2.57 (2H, CH_2 dopamine), 3.28 (2H, CH_2 dopamine), 4.26 (1H, CH PGA), 6.57−6.73 (3H, ArH dopamine). ^{13}C NMR (D_2O, δ ppm): 26.84 (CH_2 β PGA), 31.87 (CH_2 γ PGA), 33.75 (CH_2 dopamine), 40.63 (CH_2 dopamine), 53.58 (CH PGA), 116.10 (ArCH dopamine), 116.53 (ArCH dopamine), 121.08 (ArCH dopamine), 131.86 (ArCH dopamine), 142.27 (ArCH dopamine), 143.81 (ArCH dopamine), 173.58 (C═O α PGA), 179.41 (C═O γ PGA).

Determination of Drug Loading. The degree of conjugation was determined by ^{1}H NMR spectroscopic analysis, by determining the ratio between the integration of the aromatic protons of dopamine and the integration of the protons in the polymer chain. Drug loading was then calculated by determining the average of three independent analyses and is expressed as percentage of dopamine (w/w).

Determination of Purity and Quantification of Free Dopamine. The purity of the conjugate was determined by HPLC (Hewlett-Packard series 1100) using an ACE C18-300 reverse phase column (250 mm × 4.6 mm, 5 μm particle size, 300 Å pore size) (Hichrom, U.K.) as the stationary phase. Separation of the conjugate from the free dopamine was obtained by isocratic elution, with a mobile phase of MeCN/H_2O 70:30 and flow rate of 1 mL/min. PGA−dopamine (1 mg/mL) was dissolved in HPLC grade water, and an amount of 5 μL was injected into the system. The total run time was 20 min. The PGA−dopamine and free dopamine elution times were 1.5 and 5.6 min, respectively. The elution of dopamine was monitored by a UV detector at 280 nm. Free dopamine was always determined to be less than 1%.

In Vitro Cell Migration (Scratch Assay). Human umbilical vein endothelial cells (HUVEC) (Lonza, U.K.) were cultured in EGM-2 (Lonza, U.K.) in standard tissue culture conditions (37 °C, humidified 5% CO_2 atmosphere). HUVECs were seeded in 12-well plates (3×10^4 cells/well), cultured for 48 h, and then serum-starved (0.1% serum) for 24 h. Cells were then treated with medium containing VEGF (10 ng/mL) (Peprotech, U.K.) and either dopamine (1 μM) or PGA−dopamine (1 μM, dopamine equiv). HUVECs were incubated with these treatments for 1 or 24 h, after which time a scratch was performed on the cell monolayers using a 100 μL pipet tip. Cells were then washed once with PBS, and fresh treatments (VEGF + dopamine or VEGF + PGA-dopamine) were added

and left until the end of the experiment. Pictures of the scratches were taken immediately after performing the scratch ($t = 0$) and 12 h later ($t =$ 12 h) (1.3M microscope digital eyepiece camera). The area not covered by the cells was quantified using ImageJ software. Data were normalized to the presence or absence of VEGF (VEGF and VEGF-free medium were set at 100% and 0%, respectively).

In Vivo Vascular Permeability (Miles Assay). Balb/c mice were injected intraperitoneally (ip) with free dopamine for 1 or 24 h (50 mg/kg), PGA−dopamine for 24 h (50 mg/kg dopamine equiv concentration), or saline (n = 4 mice/group). A modified Miles assay was performed as previously described.[18] Briefly, Evans blue dye (100 μL of a 1% solution in 0.9% NaCl) was injected into the retro-orbital plexus of the mice. Ten minutes later, an amount of 50 μL of human $VEGF_{165}$ (1 ng/μL) or PBS was injected intradermally into the preshaved back skin of the mice. Twenty minutes later, the animals were sacrificed, and an area of skin that included the entire injection site was removed. Evans blue dye was extracted from the skin by incubation with formamide for 5 days at room temperature, and the absorbance of the extracted dye was measured at 630 nm. Data are expressed as the mean ± standard error of the mean (SEM).

Statistics. Statistical significance was estimated using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was set at $p < 0.05$.

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ABBREVIATIONS USED

DIEA, diisopropylethylamine; DIC, diisopropylcarbodiimide; DMAP, dimethylaminopyridine; DMF, dimethylformamide; Glu, glutamate; HPMA, *N*-(2-hydroxypropyl)methacrylamide; HUVEC, human umbilical vein endothelial cells; NHS, *N*-hydroxysuccinimide; PGA, polyglutamic acid; VEGF, vascular endothelial growth factor

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