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Anti-angiogenic poly-L-lysine dendrimer binds heparin and neutralizes its activity

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ABSTRACT

The interaction between heparin, a polyanion, and a polycationic dendrimer with a glycine core and lysine branches Gly–$Lys_{63}(NH_2)_{64}$ has been investigated. Complexation was assessed by transmission electron microscopy, size and zeta potential measurements, methylene blue spectroscopy, and measuring the anti-coagulant activity of heparin *in vitro* and *in vivo*. Complete association between the heparin and the dendrimer occurred a 1:1 mass ratio (2:1 molar ratio or +/− charge ratio) with formation of quasi-spherical complexes in the size range of 99–147 nm with a negative zeta potential (−47 mV). Heparin–dendrimer (dendriplex) formation led to a concentration-dependent neutralization of the anticoagulant activity of heparin in human plasma *in vitro*, with complete loss of activity at a 1:1 mass ratio. The anticoagulant activity of the dendriplexes in Sprague-Dawley rats was also evaluated after subcutaneous administration with uncomplexed heparin as a comparator. The *in vivo* anticoagulant activity of heparin in plasma, evaluated using an antifactor Xa assay, was abolished after complexation. Measurement of [^{3}H]-heparin showed that both free heparin and dendriplexes were present in plasma and in organs. Such data confirmed stably the formation of dendriplexes, which could be essential in developing novel dendrimer-based anti-angiogenic therapeutics suitable in combinatory therapeutics and theranostics.

1. Introduction

Dendrimers are three-dimensional nanocontainers synthesized in a stepwise manner by attaching branching units to an emanating core [1]. Their size, molecular weight and surface functionalities can be easily controlled [1]. We have reported recently and for the first time the intrinsic anti-angiogenic activity of cationic 6th generation poly-L-lysine (PLL) dendrimer (MW 8149 Da) using a panel of *in vitro* and *in vivo* assays [2]. Systemic administration of only two doses injected intravenously at 50 mg/kg PLL-dendrimer resulted in persistent accumulation in solid tumor sites, with reduced vascularization, extensive apoptosis/necrosis within the tumor tissue and statistically significant but moderate reduction in tumor volume, in the absence of any remarkable histological or physiological abnormality. The mechanism of PLL-dendrimer's anti-angiogenic activity is not yet fully understood however previous studies have identified heparin as a potential target for anti-angiogenesis therapy by binding to protamine, an arginine-rich basic protein of 4300 Da, that leads to inhibition of angiogenic growth factor activity [3].

Heparin is a polydisperse negatively charged polysaccharide (6000–30,000 Da). The relatively high content of sulfate groups (anionic groups) in heparin is mainly responsible for the anticoagulant and pro-angiogenic characteristics of the compound [4,5]. It has also been recognized that heparin potentiates the activity of angiogenic growth factors although this mechanism is not yet clearly understood [6]. Moreover, Azizkhan and coworkers reported that heparin released by mast cells accumulates at the tumor site, enhancing the migration of capillary endothelial cells prior to ingrowth of new blood capillaries [7]. Heparin, when present in the mammalian circulatory system, functions physiologically as an anticoagulant.

The present study is based on the hypothesis that 6th generation cationic poly-L-lysine-dendrimers Gly–$Lys_{63}(NH_2)_{64}$ [2,8]

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previously reported to accumulate at the tumor site and exhibit an intrinsic therapeutic anti-angiogenic activity that has the capability to bind electrostatically to the negatively charged heparin and thus exhibit heparin neutralizing activity. We tested the hypothesis by assessing the interaction between PLL-dendrimer and heparin using Methylene blue binding assay, dynamic light scattering and assessing heparin anti-coagulant activity by anti-factor Xa assay. Complexation of heparin and PLL-dendrimer was achieved and could lead to deactivation of heparin anticoagulant activity *in vitro* and after subcutaneous administration *in vivo*.

2. Materials and methods

2.1. Materials

Unfractionated heparin sodium salt grade I-A from porcine intestinal mucosa (187 USP units/mg) (MW 6000–30,000 Da), hydrogen peroxide, isoamyl alcohol, methylene blue (Sigma, USA), Accucolor™ heparin and Accuclot™ reference plasma (Sigma Diagnostics, USA), [^{3}H]-heparin (sodium salt) (specific radioactivity 0.32 mCi/mg) (Perkin Elmer, USA), Sagatal® (Rhone Merieux, UK), BD Eclipse™ needles, 2.7 ml BD vacutainer™ tubes (Beckton Dickinson, USA), Biosol® tissue solubilizer and self-acidified Bioscint® scintillation cocktail (National Diagnostics, UK).

2.2. Synthesis of Gly–$Lys_{63}(NH_2)_{64}$ dendrimer

The synthesis of the water soluble, glycine cored, polylysine dendrimer bearing 64 surface amino groups and employed in this study (MW 8149 Da) has been described in detail [8].

2.3. Preparation of heparin dendriplexes

The dendriplexes were formed spontaneously by mixing equal volumes (1 ml) of heparin (1 mg/ml) and the dendrimer (0.5, 1, 2, 3, 4 and 5 mg/ml) in aqueous solution, followed by gentle shaking of the colloidal dispersions on a platform shaker at 30 rpm for 1 h.

2.4. Transmission electron microscopy, size and zeta potential measurement of the dendriplexes

Heparin dendriplexes were examined by transmission electron microscopy (TEM). A drop of the suspension was placed on a grid with a support film of Formvar/carbon previously glow discharged (Emitech). Excess material was blotted off with a filter paper and the dendriplexes negatively stained with 1% uranyl acetate, prior to viewing on a Philips CM 120 Bio Twin transmission electron microscope (Einhoven, Netherlands) using a lab 6 emitter and 120 KV. Images were captured on Kodak SO-163 negative film and printed on Ilford multi-grade paper.

For size measurements, the mixtures were diluted 5 times in deionised water and sized by photon correlation spectroscopy (PCS) using a PCS 4700 Malvern submicron particle analyzer (Malvern Instruments, Malvern, UK, He–Ne laser, 90° angle of measurement). Measurements were repeated for 7 consecutive days and again at 14 days, to check for aggregation. The zeta potentials of the systems were measured using a Zetasizer 3000 (Malvern Instruments, Malvern, UK) using dispersions (2 ml) diluted 5, 10 or 20 times, depending on the original dendrimer concentration. The average of 3 measurements was used and results expressed as a Z-average (nm) ± S.D. and zeta potential (mV) ± S.D.

2.5. Methylene blue spectroscopy

A solution containing heparin–methylene blue (MB) complex was titrated with the dendrimer. A heparin–MB complex was prepared by mixing MB solution (1 ml, 20 μM), heparin solution (0.5 ml, 2.7 μM) and the calculated volume of dendrimer solution (122.7 μM) to achieve a total volume of 2 ml. MB, heparin and dendrimer final concentrations were 10, 0.725 and 0.16–10 μM, respectively. Absorption spectra for MB–heparin, before and after titration with the dendrimer, were obtained between 400 and 800 nm at a scan rate of 1200 nm/min using a plastic cuvette in a Beckman DU® 650 Spectrophotometer (USA). A_{664}/A_{568} ratio was used to calculate the ratio at maximum association. The experiments were repeated in quadruplicate.

2.6. Estimation of the anticoagulant activity of dendriplexes in vitro

The anticoagulant activity of heparin and the heparin dendriplexes *in vitro* was tested by antifactor Xa assay, using an Accucolor™ heparin kit (micro-plate method). Accucolor™ heparin is intended for the quantitative determination of therapeutic heparin in human plasma. Lyophilized human antithrombin III (AT-III), bovine factor Xa, and factor Xa substrate were reconstituted in deionized water as directed by the manufacturer. Lyophilized Accuclot™ reference plasma was reconstituted in deionized water immediately before use.

A solution of heparin in saline was prepared as a stock solution (8 USP unit/ml or 44 μg/ml) and a calibration curve of heparin (0.1–0.8 IU/ml) obtained by serial dilution of the stock in standard human plasma. Heparin dendriplexes were prepared in standard human plasma, at dendrimer/heparin (0.1–0.8 IU/ml) mass ratios of 0.1, 0.5, 1, 2, 3, 4 and 5:1. An additional 1:2 dilution of all standards and dendriplexes was made by diluting 100 μl of the plasma standard with 100 μl saline before running the assay. To run the assay, AT-III (75 μl) was dispensed to individual wells of a 96-well microstate plate and incubated with the diluted standard (25 μl) and factor Xa (75 μl), with factor Xa substrate (75 μl) subsequently added and incubated at 37 °C for 2, 1 and 10 min after each addition, respectively. The reaction was stopped with 20% acetic acid (75 μl). A_{405} was blanked using these reagents in the following order; acetic acid, AT-III, plasma, factor Xa and the substrate, and then measured using a Dynex MRX Microplate Reader 133 (Dynex, UK). When A_{405} was plotted against heparin concentration (units/ml), the anticoagulant activity was inversely proportional to A_{405}. Measurements were carried out in triplicate.

2.7. Estimation of the anticoagulant activity of dendriplexes in vivo in the rat

2.7.1. Animals

Female, Sprague-Dawley rats (176.4 g ± 10.5 g) (B and K Universal Ltd., UK) were caged in groups of 3–8 with free access to water. A temperature of 19–22 °C was maintained, with a relative humidity of 45–65%, and a 12 h light/dark cycle. Animals were acclimatized for 7 days before each experiment and were fasted for 12 h before dosing. All procedures followed the 1989 UK Home Office "Code of Practice for the Housing and Care of Animals used in Scientific Procedures".

2.7.2. Dosing of animals

A subcutaneous heparin dose was used as recommended by Borchard et al. [9]. The oral heparin dose was determined from dose-response studies conducted by Leone-Bay et al. [10]. Dose volumes were chosen as recommended by Diehl et al. [11].

Subcutaneous heparin administration: 20 rats were randomized into 5 groups of 4 animals each and injected subcutaneously with a single dose of [^{3}H]-heparin (10 mg/Kg, 1 μCi, 0.2 ml) in the dorsal neck region. Groups were sacrificed at 0 (control), 1, 3, 6 and 24 h after administration.

Subcutaneous dendriplexes administration: 20 rats were randomized into 5 groups of 4 animals each and injected subcutaneously in the dorsal neck region with a single dose of [^{3}H]-heparin complexed with dendrimer (10 mg/Kg heparin and 30 mg/Kg dendrimer, 1 μCi, 0.2 ml). Groups were sacrificed at 0 (control), 1, 3, 6 and 24 h after administration.

Oral heparin administration: 20 rats were randomized into 5 groups of 4 animals each and gavaged with a single dose of [^{3}H]-heparin (100 mg/Kg, 1 μCi, 2 ml). Groups were sacrificed at 0 (control), 1, 3, 6 and 24 h after administration.

2.7.3. Blood collection and radioactivity in plasma and organs

At the scheduled time points (0, 1, 3, 6 and 24 h) post dosing, animals were anesthetised by intraperitoneal (ip) injection of pentobarbitone sodium B.P. (72 mg/rat) (Sagatal®, 60 mg/ml). Blood was withdrawn from the abdominal aorta using BD Eclipse™ needles and collected into 2.7 ml BD Vacutainer™ tubes containing 0.129 M sodium citrate (3.8%, 9:1 blood:sodium citrate ratio) as anticoagulant. The tubes were transferred onto ice, centrifuged (2500 g, 10 min) at room temperature, and the plasma immediately harvested and stored at −20 °C. After sacrifice, the heart, lungs, liver, spleen, kidneys, stomach, small intestine and large intestine were removed and homogenized in deionised water for 3 min, intermittently, using a tissue homogenizer (Model PT-10, Polytron, Switzerland). All samples were solubilised before radioactivity counting; an aliquot of 0.6 ml of plasma or 200 μg of each tissue homogenate was solubilised with 1.0 ml of Biosol® (National Diagnostics, UK) tissue solubiliser, shaken overnight at 55 °C, combined with 17 ml of the self-acidified Bioscint® scintillation cocktail (to eliminate chemiluminescence), and kept in a dark cold room at 4 °C for 72 h before counting in a LS6500 Multi-Purpose Scintillation Counter (Beckman, USA). Colored samples were decolorized with 200–400 μl of 30% hydrogen peroxide, and with 200–400 μl of isoamyl alcohol added to stop foaming. Rat plasma volume was calculated to be in the range of 6.1–7.6 ml based on the formula of 36–45 ml plasma per Kg of rat body weight [12]. Results were expressed as the percent of the injected dose per organ, or the percent of the injected dose per g tissue ± S.D. (n=4). Plasma volume rather than plasma weight was used in all calculations.

2.7.4. Antifactor Xa assay

The standard curve of free heparin (IU/ml) was established using the procedure described above in the *in vitro* study, except that rat plasma was substituted for human plasma, and 50 μl of undiluted rat plasma was used instead of 25 μl of diluted human plasma. A standard curve was prepared by spiking heparin (0.1–1.6 IU/ml) into control pooled platelet-free rat plasma. Test plasma samples were assayed within 2 h of collection, or stored at −20 °C and thawed once at 37 °C, 30 min before use.

3. Results and discussions

3.1. Shape, size and zeta potential measurement of the dendriplexes

Heparin is a polydisperse polyanionic copolymer composed of alternating D-glucuronic acid, which is frequently O-sulfated at C2, and D-glucosamine-N-sulfate with an additional sulfate group on C6 linked via α-1,4 glycosidic linkage; molecular weights range from 6000 to 30,000 Da. The heparin used in this experiment had a mean number molecular weight within the range of 17,000–19,000 Da, as specified by the manufacturer (Sigma, UK). Gelman and Blackwell [13] reported heparin to have 2.33 sulfate groups per disaccharide and showed a maximum interaction between the linear poly(lys) and the heparin at a 2.3:1 amino acid:disaccharide ratio; the complex adopted an α-helical conformation.

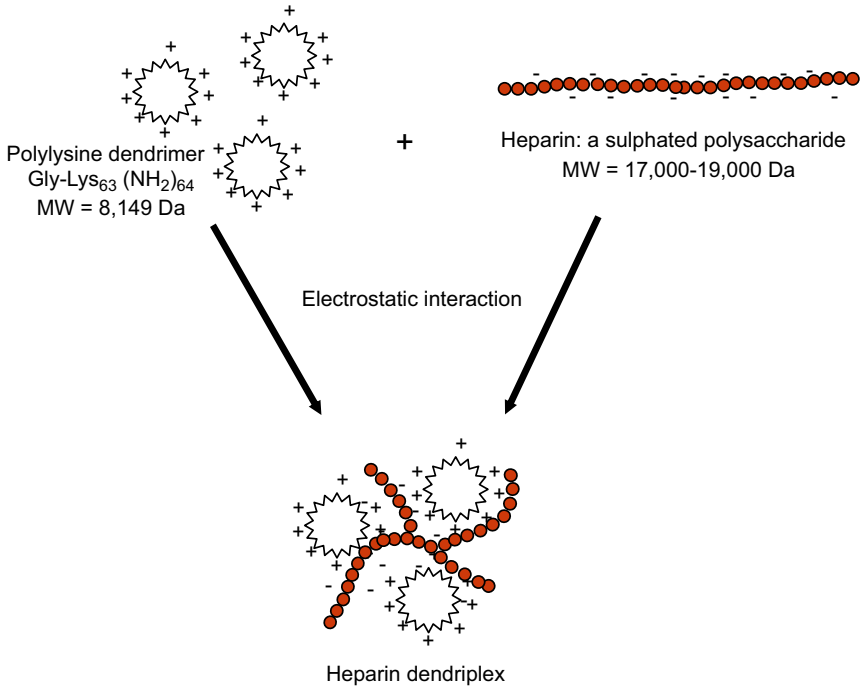

Fig. 1. Heparin dendriplexes are spontaneously formed as a result of electrostatic interactions by mixing heparin and the cationic dendrimer in aqueous solution, as illustrated. The number of charges assigned per heparin or dendrimer molecule in the illustration is only a schematic representation and does not represent the actual charge per molecule.

Heparin–dendrimer complexes, or dendriplexes, were spontaneously formed by mixing heparin and the cationic dendrimer in aqueous solution, the formation presumably occurring via electrostatic interactions, as illustrated in Fig. 1. The spherical aggregates shown by TEM in Fig. 2A and B were not observed in heparin or dendrimer solutions alone, indicating that the configuration of the aggregates was specific to dendriplex formation. The mean intensity hydrodynamic diameter of the dendriplexes, prepared at varying dendrimer to heparin mass ratios, and measured by PCS, ranged from 99.0 ± 4.0 nm to 147.0 ± 3.0 nm (Table 1). Size was monitored and shown to be generally stable for up to 14 days (Fig. 2C). However, the dendriplexes exhibited a negative zeta potential (−47 mV) at a 1:1 mass ratio (2:1+/− charge or molar ratio), despite complete association (as will be discussed in the MB/heparin assay). The zeta potential was

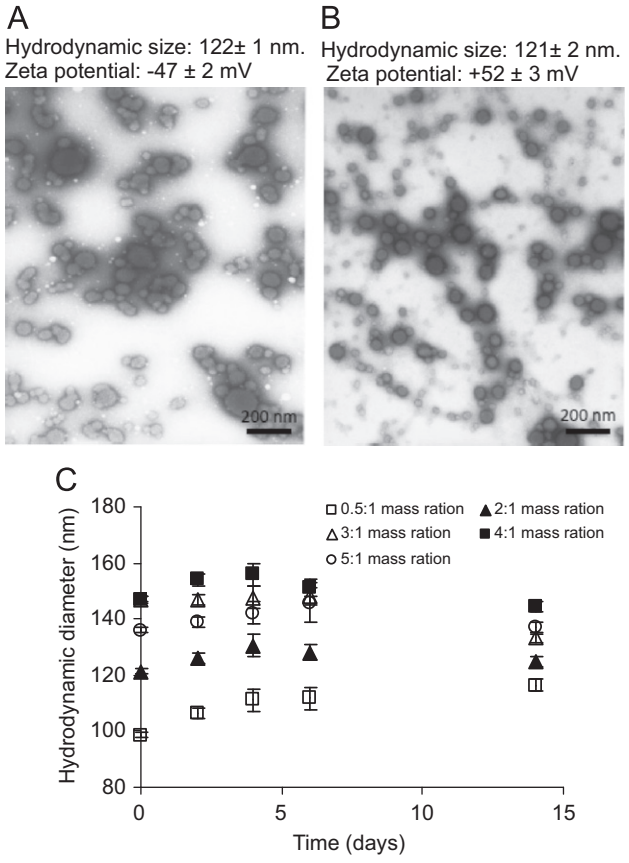

Fig. 2. Transmission electron micrographs of dendriplexes showing quasi-spherical aggregates in the nano-size range at 1:1 dendrimer:heparin mass ratio (A) and at 2:1 dendrimer:heparin mass ratio (B). (C) The mean Z-average diameter (nm) of dendriplexes as a function of time (in days) at various dendrimer:heparin mass ratios.

Table 1
Z-average diameter (nm) and zeta potential (mV) of dendriplexes formed on day 0, from various dendrimer:heparin ratios.

Dendrimer: heparin mass ratio	Dendrimer: heparin molar ratio[a]	+/− charge ratio[b]	Z-average diameter (nm) ± S.D.[c]	Zeta potential (mV) ± S.D.[c]
0.5:1	1:1	1:1	99.0 ± 4.0	−38.0 ± 1.6
1:1	2:1	2:1	122.0 ± 1.0	−47.0 ± 2.0
2:1	4:1	4:1	121.0 ± 2.0	+52.0 ± 3.0
3:1	6:1	6:1	147.0 ± 3.0	+55.0 ± 4.0
4:1	8:1	8:1	147.0 ± 1.5	+56.0 ± 2.0
5:1	10:1	10:1	136.0 ± 2.0	+60.0 ± 2.0

[a] Based on the assumption that heparin has an 18,000 Da average molecular weight.

[b] Based on the assumption that there are 69 negative charges per heparin chain, and 64 positive charges per dendrimer. The +/− charge ratio was calculated using the equations:

$$+/-Ratio = 2.05 \times \frac{Dendrimer[weight]}{Heparin[weight]} \text{ or } \pm Ratio = 0.92 \times \frac{Dendrimer[moles]}{Heparin[moles]}$$

[c] Mean ± S.D. (n=3).

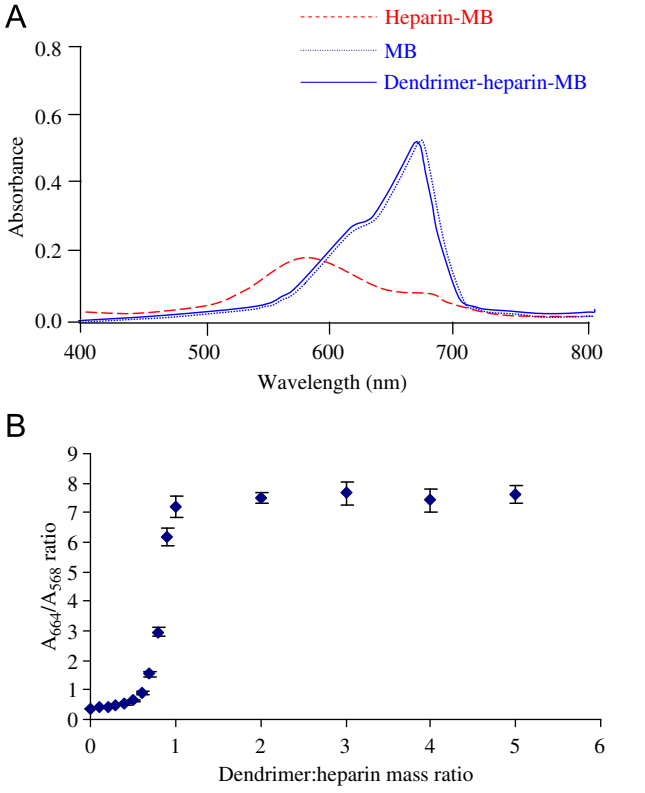

Fig. 3. (A) UV/vis spectra of free methylene blue (MB) (λ_{max}=664 nm) and MB–heparin (λ_{max}=568 nm). Upon dendrimer addition, the MB–heparin λ_{max} shifted to 664 nm indicating MB release from heparin due to dendrimer:heparin complexation. (B) Titration of a MB (10 μM)–heparin (0.725 μM) mixture with the dendrimer (0.16–10 μM). A maximum A_{664}/A_{568} ratio was obtained at a 2:1+/− charge ratio (1:1 or 2:1 dendrimer:heparin mass or molar ratio, respectively). This is considered to be the ratio at which a maximum dendrimer:heparin association occurred.

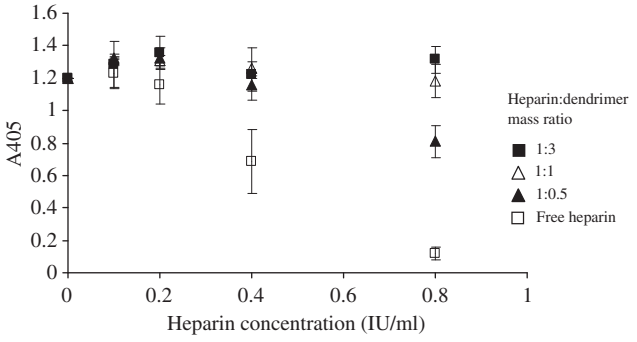

Fig. 4. Heparin anticoagulant activity *in vitro* was measured using an antifactor Xa assay. A_{405} is inversely proportional to the active heparin concentration. Heparin activity was reduced as the dendrimer concentration increased. Heparin activity (0.8 IU/ml) began to decrease (and A_{405} increase) as the dendrimer:heparin mass ratio increased from 0.5:1 to 3:1, with complete neutralization at a 1:1 ratio. Symbols are the means of triplicate assays.

positive (+52 mV) at a 2:1 mass ratio (4:1+/−charge or molar ratio) (Table 1).

The dendrimer/heparin charge ratio was calculated based on the assumption of 18,000 Da average molecular weight for heparin and 2.3 sulfate groups per disaccharide (Table 1). The reason that the maximum association in dendrimer/heparin systems occurred at 2:1+/−, compared to 1:1+/− in linear polylysine/dendrimer systems [13] may be due to the non-availability of all the amino groups of the dendrimers for interaction with sulfate groups. This could be related to steric hindrance of the terminal amines with the hyperbranched dendrimer structure, or to pH-dependent ionization of the dendrimer amino groups. A further possibility is that the heparin used in the present study was a polydisperse mixture of heparin chains, whereas the assumption in calculating the +/−charge ratio was based on a single molecular weight.

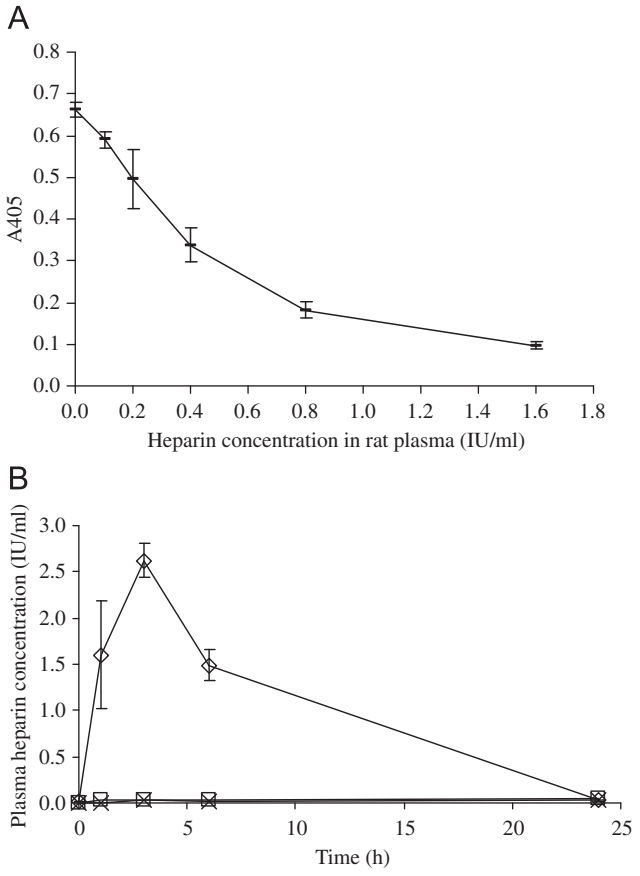

Fig. 5. (A) Heparin calibration was carried out using heparin spiked (0.0–1.6 IU/ml) control pooled platelet-free rat plasma, and an antifactor Xa assay ($n=4$; symbols are means, ± S.D.). (B) Active heparin plasma concentration (IU/ml) in Sprague-Dawley rats treated by subcutaneous injection with [^{3}H]heparin (10 mg/Kg) (◇), or [^{3}H]heparin dendriplex (10 mg/Kg heparin and 30 mg/Kg dendrimer) (□). Oral [^{3}H]heparin (100 mg/Kg) was used as a negative control (×). There were 4 animals in each treatment group at each time point (0 control, 1, 3, 6, and 24 h post-administration).

3.2. Methylene blue/heparin assay

To confirm that an electrostatic-type interaction was the driving force for dendriplex formation, MB spectroscopy was employed. MB is a cationic metachromatic dye with an affinity for polyanions such as heparin [10]. Unbound MB has a λ_{max} of 664 nm whereas MB bound to heparin (MB–heparin) has a λ_{max} of 568 nm (Fig. 3A). Dendrimer addition to MB–heparin caused a shift in λ_{max} from 568 to 664 nm. MB and MB–dendrimer exhibit the same λ_{max} of 664 nm, which excludes any interaction between MB and the dendrimer. MB spectroscopy (A_{664}/A_{568} ratio) was used to identify the ratio at which maximum dendrimer–heparin association occurred. First, the optimum heparin concentration required to produce a minimum $A_{664/568}$ ratio was found experimentally, i.e. all MB molecules (10 μM) were bound to heparin with no excess heparin (0.725 μM) in the solution; excess free heparin in the medium would give an inaccurate dendrimer/heparin association ratio. The MB–heparin mixture was titrated with dendrimer (0.16–10 μM). A maximum A_{664}/A_{568} ratio was obtained at a 1:1 mass ratio (2:1+/−charge ratio or molar ratio) (Fig. 3B). This result agrees with the zeta potential measurement study, which showed a negative zeta potential (−47 mV) at this molar ratio, which then became positive (+52 mV) when the ratio was increased; this would indicate the presence of excess dendrimer on the complex surface at higher molar ratios.

3.3. In vitro anticoagulant activity of heparin dendriplexes

Antithrombin III (AT-III) is a natural inhibitor of thrombin, factor Xa and other coagulation proteases in plasma. The rate of inhibition by AT-III is slow, but the rate can be increased several thousand times by the presence of heparin. Thus the antifactor Xa assay is a useful test to evaluate the anticoagulant activity of heparin. A commercial antifactor Xa assay kit was used to estimate the residual anticoagulant activity of heparin. Since both factor Xa and AT-III are present in excess in the assay kit, the rate of factor Xa inhibition is directly proportional to the heparin concentration. The residual activity of factor Xa, as measured by the absorbance of its chromogenic substrate at 405 nm, is

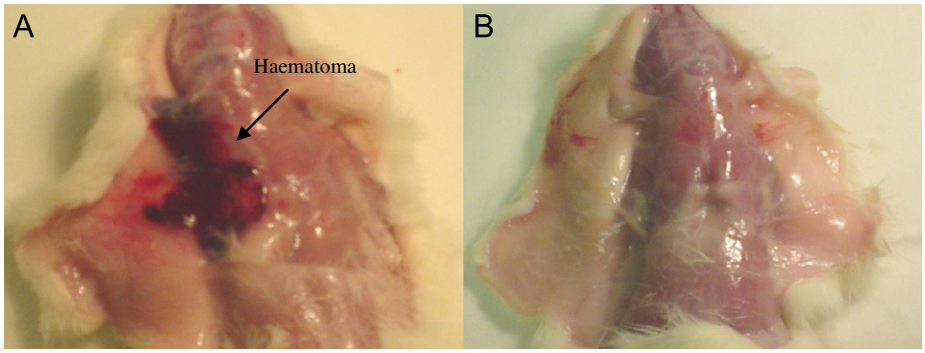

Fig. 6. (A) Subcutaneous injection site in the dorsal neck region at 1 h after the administration of [^{3}H]heparin (10 mg/Kg). (B) Injection site at 1 h after the administration of [^{3}H]heparin dendriplex (10/30 mg/Kg). In (A) there is evidence of hematoma formation, while there is no evidence of hemorrhage in (B). In each image the dorsal skin in the neck region has been deflected to the left and right of the mid line; the cranium is towards the upper part of each image.

Table 2
[^{3}H]heparin and [^{3}H]heparin dendriplex administration by subcutaneous injection in the rat; percentage of administered dose per organ at 1, 3, 6 and 24 h after treatment[a].

Organ	1 h	3 h	6 h	24 h
[^{3}H]heparin				
Plasma	1.73 ± 0.54	0.83 ± 0.19	0.56 ± 0.09	0.45 ± 0.04
Heart	0.11 ± 0.04	0.12 ± 0.09	0.07 ± 0.01	0.10 ± 0.04
Lung	0.18 ± 0.06	0.15 ± 0.03	0.14 ± 0.02	0.12 ± 0.01
Liver	1.14 ± 0.17	0.87 ± 0.05	0.84 ± 0.02	0.86 ± 0.18
Spleen	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.06	0.04 ± 0.03
Kidney	1.87 ± 0.95	1.47v0.29	1.49 ± 0.69	1.16 ± 0.10
Stomach	0.14 ± 0.05	0.18 ± 0.07	0.28 ± 0.09	0.12 ± 0.05
Small intestine	0.84 ± 0.25	0.90 ± 0.38	0.57 ± 0.09	0.43 ± 0.06
Large intestine	0.37 ± 0.02	0.36 ± 0.07	0.42 ± 0.13	0.34 ± 0.06
[^{3}H]heparin dendriplex				
Plasma	1.26 ± 0.04	0.82 ± 0.15	0.68 ± 0.12	0.35 ± 0.06
Heart	0.06 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.01
Lung	0.08 ± 0.01	0.09 ± 0.00	0.09 ± 0.02	0.06 ± 0.01
Liver	0.90 ± 0.07	1.13 ± 0.13	1.57 ± 0.46	1.62 ± 0.36
Spleen	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
Kidney	0.87 ± 0.03	0.70 ± 0.07	0.74 ± 0.20	0.72 ± 0.12
Stomach	0.13 ± 0.06	0.09 ± 0.01	0.07 ± 0.02	0.03 ± 0.02
Small intestine	0.59 ± 0.56	0.38 ± 0.17	0.22 ± 0.02	0.17 ± 0.06
Large intestine	0.12 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.12 ± 0.03

[a] Data are means, ± S.D. There were 4 animals in each treatment group at each time point.

inversely proportional to the anticoagulant activity of heparin in plasma.

The antifactor Xa assay was employed to study the effect of complexation on the *in vitro* anticoagulant activity of heparin. The USP unit is the measure of the anticoagulant activity of a given heparin product using a USP reference standard based on units of heparin activity per mg, and the heparin used in the present work has 180 USP units/mg. It was found that when dendrimer was bound, neutralisation of anticoagulant activity occurred (Fig. 4); heparin spiked (0.8 IU/ml) in human plasma was inactive at a 1:1 heparin:dendrimer mass ratio. This further confirmed the result obtained by MB spectroscopy which showed that the maximum association between heparin and dendrimer also occurred at the 1:1 mass ratio. The new conformation of heparin is considered to abolish the negative charge and make it inaccessible to AT-III and the coagulation proteases (i.e. prothrombin III and factor Xa), a process, which inactivates anticoagulant activity.

3.4. *In vivo anticoagulant activity of heparin and heparin dendriplexes after subcutaneous administration in the rat*

Heparin dendriplexes demonstrated no anticoagulant activity *in vitro*, however it was considered of interest to determine if the behavior of such macromolecular complexes could allow activity in complex biological systems; events such as partitioning

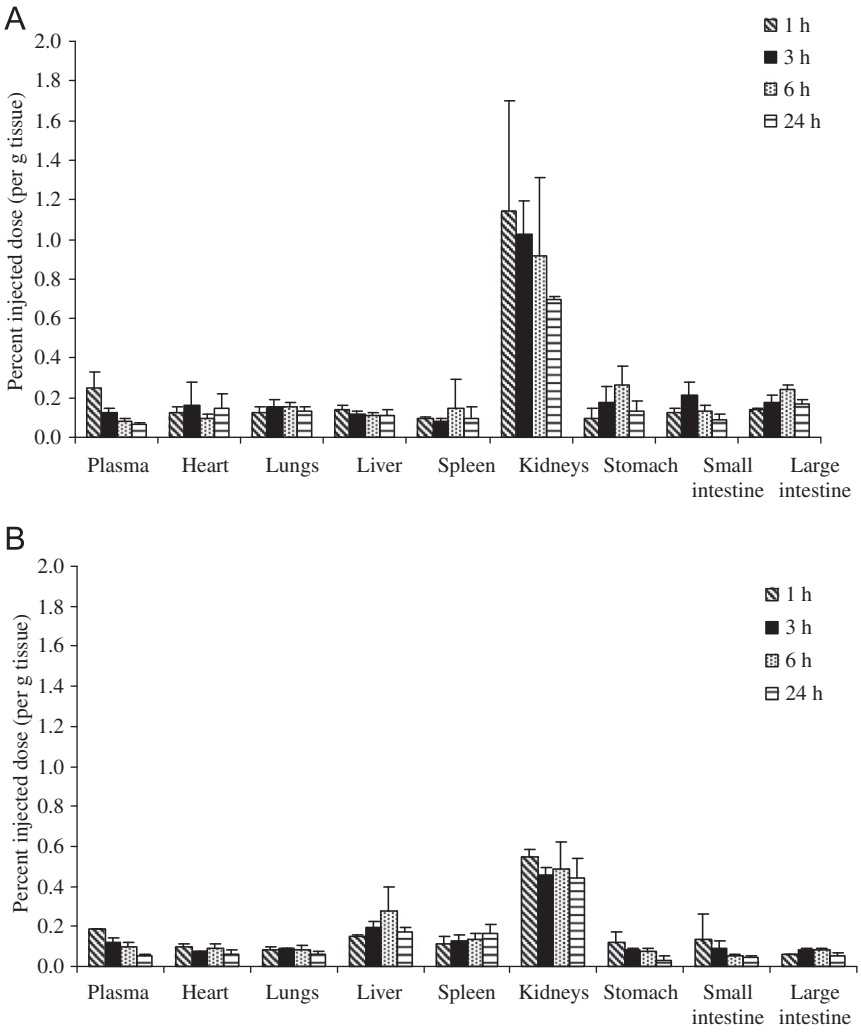

Fig. 7. Percentage of injected dose per g tissue (mean ± S.D.) of [^{3}H]heparin (A), and [^{3}H]heparin dendriplex (B), after subcutaneous injection in the rat, at 1, 3, 6 and 24 h after treatment.

between the dendrimer and heparin binding peptides may occur, as the binding of heparin to heparin binding peptides has been reported in plasma following intravenous administration [14]. Other effects that could occur *in vivo* include an interaction with extracellular matrices after subcutaneous injection and/or interactions with intracellular components following cellular uptake (e.g. susceptibility to endosomal escape following endocytosis).

To investigate whether heparin was able to regain anticoagulant activity *in vivo*, rats were injected subcutaneously with free or complexed heparin. Heparin anticoagulant activity in platelet-free rat plasma was estimated at 0, 1, 3, 6 and 24 h post-injection using the antifactor Xa assay. The heparin calibration curve in platelet-free pooled rat plasma was plotted (Fig. 5A) and the active heparin concentration in the plasma determined (Fig. 5B). It can be seen (Fig. 5B) that heparin injected subcutaneously at a dose of 10 mg/Kg was therapeutically active up to 6 h post-administration and complete loss of its activity was observed at 24 h time point. However, heparin dendriplexes at a 1:3 heparin:dendrimer mass ratio (10/30 mg/Kg) (a ratio above maximum association), resulted in an inactive complex *in vivo*; this lack of activity compared with the oral heparin-treated groups. At autopsy, gross observations were made on the injection site in animals treated with heparin and heparin dendriplexes. In animals injected with heparin there was evidence of subcutaneous hematoma formation (i.e. localized extravasated blood). There was no evidence of hemorrhage at the injection site in animals treated with heparin dendriplex (Fig. 6).

It was considered that the lack of anticoagulant activity *in vivo* in rats treated with heparin dendriplexes may have been due to the precipitation of the aggregates at the site of subcutaneous injection. Therefore, an estimation of the total heparin concentration (both active and inactive) was carried out by measuring the radioactivity of free heparin and heparin dendriplexes in the plasma and major organs, at a series of time points after subcutaneous administration. Data obtained from radioactivity measurements (Table 2) demonstrated that the administration of both free and complexed heparin lead to the identification of radioactivity in the plasma and the major organs. This finding implied that heparin dendriplex inactivity could be attributed to complexation and not to precipitation at the site of administration. Results from biodistribution studies (Fig. 7) show that both heparin and heparin dendriplexes have the highest levels in the kidney, with reductions in plasma and kidney levels as a function of time.

The reported results confirm that the polycationic hyperbranched poly-L-lysine dendrimer has great affinity to the polyanionic heparin which could be of interest in developing further and in more predictable manner new heparin-binding antiangiogenic therapeutics. Furthermore, it further indicates heparin binding to poly-L-Lysine dendrimer could be one of the postulated mechanisms behind the dendrimer intrinsic anti-angiogenic activity.

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