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Regulated Heparin Release using Novel Quantum Dots for Potential Application to Vascular Graft Engineering

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This paper presents a new approach in the field of controlled drug delivery systems using a novel quantum dot (QD). We developed a system for polymeric microencapsulated drugs which is conjugated to near infrared (NIR) absorbing quantum dots and tested the feasibility of burst release of a model drug, heparin, from microcapsules triggered by irradiation. We have shown the burst release of heparin from microcapsules can be achieved by irradiation. This system is designed to externally modulate drug release in response to physiological needs by control of the intensity and period of irradiation. These results suggest that QD can be a key component to be used for triggering the release of drugs for various clinical applications. We further investigated the heparin incorporation into and release from decellularized blood vessels for potential application of functionalized heparin for vascular graft engineering.

Keywords: Polymeric controlled release, external regulation, quantum dot, NIR energy, heparin, vascular graft, tissue engineering

1 Introduction

Polymeric controlled drug delivery has evolved from the need for prolonged and better control of drug administration. The controlled drug delivery system can maintain the drug in the desired therapeutic range for an extended period of time with a single dose. Localized delivery of the drug to a specific site of the body reduces the need for follow up care, preserves medications and increases the patient comfort (1–4).

Nevertheless, there are a number of clinical situations where such an approach may not be sufficient. These include the delivery of insulin for patients with diabetes mellitus, anti-arrhythmics for heart rhythm disorders, gastric acid inhibitors for ulcer control, nitrate for patients with angina pectoris, as well as immunization, cancer chemotherapy and suppressing thrombosis after surgical operation (5–9).

For the past decades, technologies have been developed to externally trigger the drug release according to physiological needs. Magnetic, ultrasonic, thermal, and electric sources were used for pulsed delivery (10–16). With recent advances in and the unique properties of quantum dots (QD), we developed a new approach for external regulation of drug release by using a near infrared (NIR) absorbing QD. NIR QD absorb NIR energy and become heated when they were irradiated (17).

Heparin is a potent anticoagulant agent administered systemically following vascular surgery to prevent acute thrombosis. Low molecular weight heparin (LMWH) is also used in treatment and is proven to confer more advantages than unfractionated heparin (18, 19). Site-specific delivery of anti-thrombogenic factors such as heparin from a vascular graft should provide an efficient means for preventing thrombosis and suppressing the over proliferation of the smooth muscle cells in the vessel and thus would ensure the function of engineered vessel.

Conjugation of heparin to a synthetic vascular scaffold offers a localized release system for heparin. However, immobilization of heparin to the scaffolds results in slow release that can not properly prevent thrombus formation normally after surgical operation. In order to suppress the acute thrombosis, burst release of heparin is required. In this study, we developed a system of

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microencapsulated heparin conjugated to NIR absorbing QD, and tested the feasibility of burst release of heparin from the polymeric microcapsules triggered by NIR irradiation.

We further studied *in vitro* and *in vivo* heparin release from the functionalized vascular scaffolds, and assessed the ability of the heparin incorporated vascular graft in inducing the anticoagulation.

2 Experimental

2.1 Materials

All reagents were used without further purifications. Poly(D,L-lactide-*co*-glycolide) (PLGA) (Mw 110,000 g/mol, 50:50 by mol ratio of lactide and glycolide, Resomer® RG506) was purchased from Boehringer-Ingelheim (Ingelheim, Germany).

Poly(ε-caprolactone) (PCL) (Mw 110,000 g/mol), poly(vinyl alcohol) (PVA), and phenylene diamine (PDA) were obtained from Sigma (St. Louis, MD, USA). QDs were obtained from Evident Technologies (Troy, NY, USA). QDs used for the drug burst measurements were red CdSe with a primary excitation of 627 nm. Green CdSe QDs with a primary excitation of 522 nm were used for fluorescent imaging of the microcaplsules. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfonated N-hydroxysuccinimide (sulfo-NHS) were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Heparin (Lovenox®, enoxaparin sodium) was purchased from Aventis Pharmaceuticals Inc. (Bridgewater, NJ, USA).

2.2 Functionalization

EDC (10 mg) and sulfo-NHS (2 mg) were added to 5 mL (0.05 mg/mL) of carboxylated QDs (red) in aqueous solution under gentle stirring for 1 h at room temperature. EDC activated heparin (30 mg/20 μl) was prepared according to the same EDC and NHS method. In order to conjugate quantum dots and heparin, 5 mg PDA was added to the activated quantum dots and heparin solutions under stirring for 2 h at room temperature. The quantum dot-heparin (QD-heparin) conjugation can be quenched by adding an equal volume of 1 M Tris buffer solution (pH 7.4) and stored in 4°C (Fig. 1).

2.3 Encapsulation

Microencapsulation of QD-heparin was performed by double emulsion. Briefly, 4 mL of internal aqueous phase containing 30 mg QD-heparin and 10 mg bovine serum albumin (BSA, Sigma) as stabilizer was emulsified in 8 ml solution of 100 mg PLGA and 100 mg PCL in dichloromethane (Sigma). The solution was emulsified by vortexing for 5 min at room temperature. This W/O dispersion was diluted to 200 ml of 1% (w/v) aqueous PVA solution under stirring for 4 h at room temperature. The microcapsules were washed several times with deionized water and then lyophilized overnight. The morphology

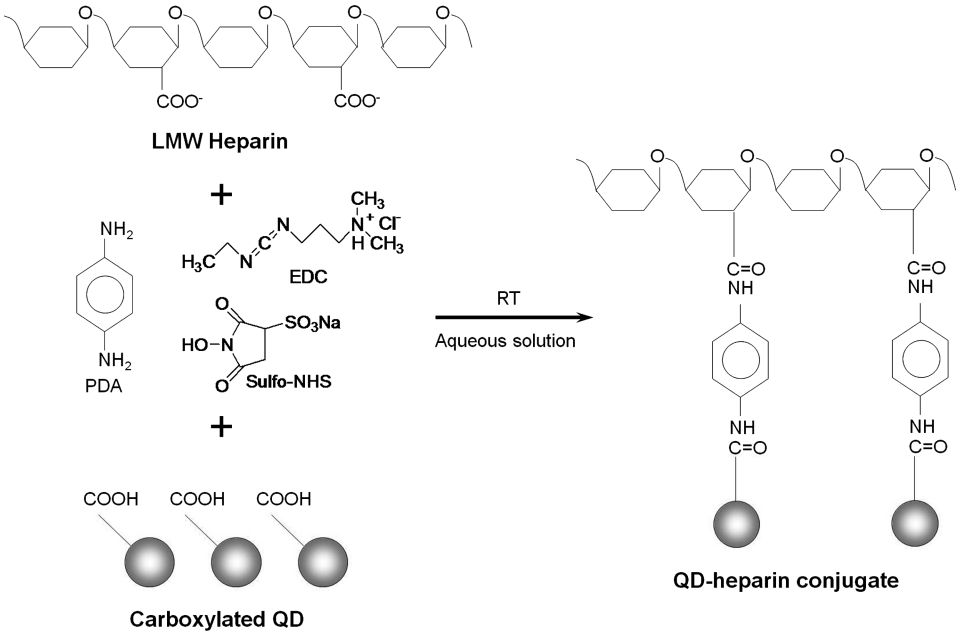


Fig. 1. Diagram showing conjugation of heparin on quantum dots.

of microspheres was examined with scanning electron microscopy (SEM; S-2600N, Hitachi, Japan).

2.4 Heparin Release using IR Irradiation of the Quantum Dot

In order to evaluate the burst release of heparin, 0.55 mg of PLGA microcapsules containing QD-heparin were suspended in 2 ml of PBS (phosphate buffered saline). The solution was irradiated for 0, 10 and 30 min using an AM1.5 solar simulator at 75 mW/cm^2. On days 1, 3 and 5, the samples were then cooled to 4°C, centrifuged at 4500 rpm for 20 min and filtered (0.45 μm pore size) to remove any microcapsules for the optical measurements. Luminescence measurements were performed using an argon ion laser (514.5 nm at 400 mW/cm^2) as the excitation source and spectra were collected using a CCD spectrophotometer with an integration time of 40 sec.

2.5 Analysis of Heparin Released from Microcapsule

The heparin concentration within the microcapsule and heparin released from the microcapsule were evaluated by measuring the antifactor Xa activity with a chromogenic substrate using STA®-Rotachrom® Heparin kit from Diagnostica Stago (Parsippany, NJ, USA). The assay had a coefficient of variation of $< 5\%$ at a detection limit of 2 ng/ml.

2.6 Decellularization of Blood Vessel

In order to obtain collagen-rich tubular scaffolds we chose to process segments of porcine iliac, femoral and carotid arteries. These segments possessed an internal lumen size of 3–4 mm and were approximately 4 cm long. Porcine blood vessel was harvested from sacrificed animals and rinsed with distilled water. Following the methods for decellularization (20, 21), under continuous agitation, the blood vessel was washed with 0.05% trypsin for 24 h. And then the blood vessel was washed with 10% fetal bovine serum to inactivate trypsin for overnight. The blood vessel was further washed with 1% Triton X-100 and 0.1% ammonium hydroxide for 3 days. Arterial segments were washed with distilled water for 24 h and rinsed with PBS for overnight. The grafts were lyophilized and sterilized with ethylene oxide.

2.7 Heparinization of Decellularized Blood Vessel

2.7.1. *Heparinization* 1

30 mg heparin was incubated in 20 mM EDC and 10 mM sulfo-NHS in PBS for 2 h at room temperature and then the decellularized blood vessel (5 cm of length and 3∼5 mm of diameter) was immersed in heparin-EDC solution for 2 h at room temperature. The resulting heparinized samples were rinsed in PBS to remove residual EDC for 24 h at room temperature (Fig. 2).

2.7.2. *Heparinization* 2

The decellularized blood vessel was incubated in 2 mg/mL Poly(L-lysine) (PLL: mol wt 30,000–70,000, Sigma) solution for 2 h at 37°C. The PLL-adsorbed blood vessel was immersed in 15 mg/mL heparin solution for 1 h at room temperature (Fig. 3).

2.8 Incorporation of Quantum Dot-Heparin Polymer Microspheres into Decellularized Blood Vessels

To attach the QD-heparin loaded polymeric microspheres onto the blood vessel, 100 mg PLGA (Mw; 8,000) was dissolved in 10 mL dichloromethane. The blood vessel was immersed into 1% PLGA solution and then 10 mg

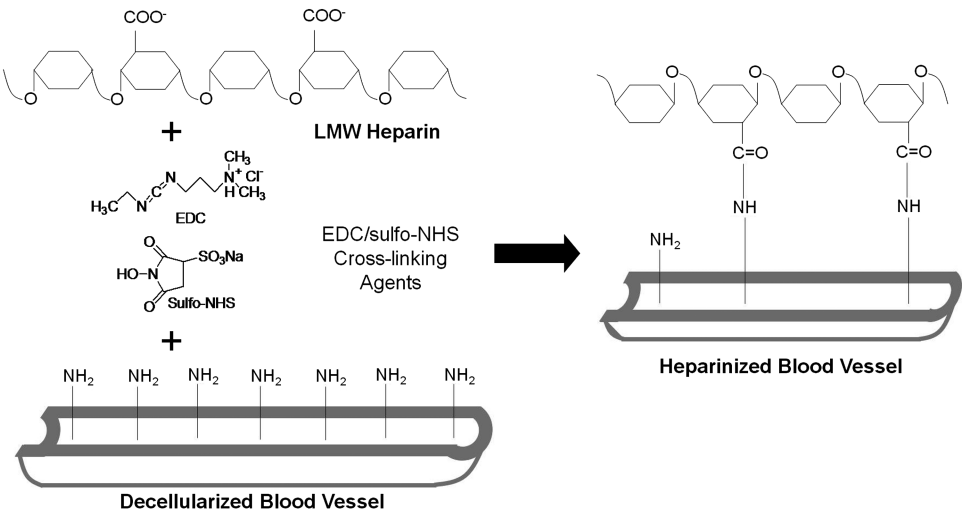


Fig. 2. Heparinization of decellularized blood vessel using EDC/NHS crosslinking agent.

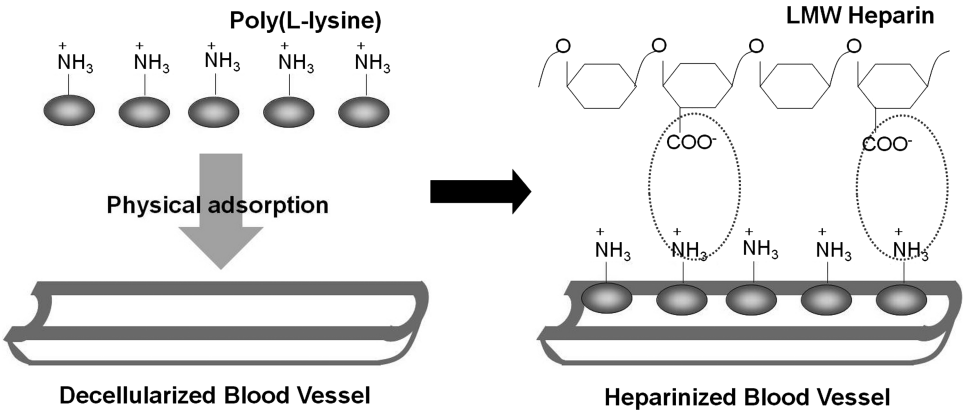


Fig. 3. Heparinization of decellularized blood vessel using Poly(L-lysine).

microspheres were attached to the PLGA solution treated blood vessel. The microspheres-attached blood vessel was dried in a fume hood for 2 h at RT and stored in vacuum desiccators.

2.9 Evaluation of Immobilized Heparin by Toluidine Blue Staining

Toluidine blue belongs to the thiazine dyes that are especially suited for nuclear staining of histological material. Toluidine blue can be used for the demonstration of metachromasia, which is typical of very dense structures (spaced less than 0.4 nm) of negative valences. Demonstration is possible with diluted staining solutions containing monomers of dye only, which bind to the single bond of negative valences. Uniformity of heparin immobilization of the graft was assessed with toluidine blue staining. Briefly, after incubation in PBS for 5 min, the heparinized vascular grafts were staining during 1 min using a 0.1% toluidine blue in DI water. The vascular grafts were washed in PBS (three times for 5 min) and dehydrated in a graded series of ethanol (50, 60, 70, 80, 90, and 100%) for 5 min each.

2.10 Flow System for Heparinized Decellularized Blood Vessel

In order to study heparin release kinetics from decellularized blood vessel, a flow system consisted of circular chamber, reservoir, and peristaltic pump was used. The circular chamber has a closeable inlet and an outlet (Fig. 4). The heparinized decellularized blood vessel was mounted between inlet an outlet lines and perfused continuously for 9 days. The media were collected and analyzed by using STA® -Rotachrom® Heparin kit.

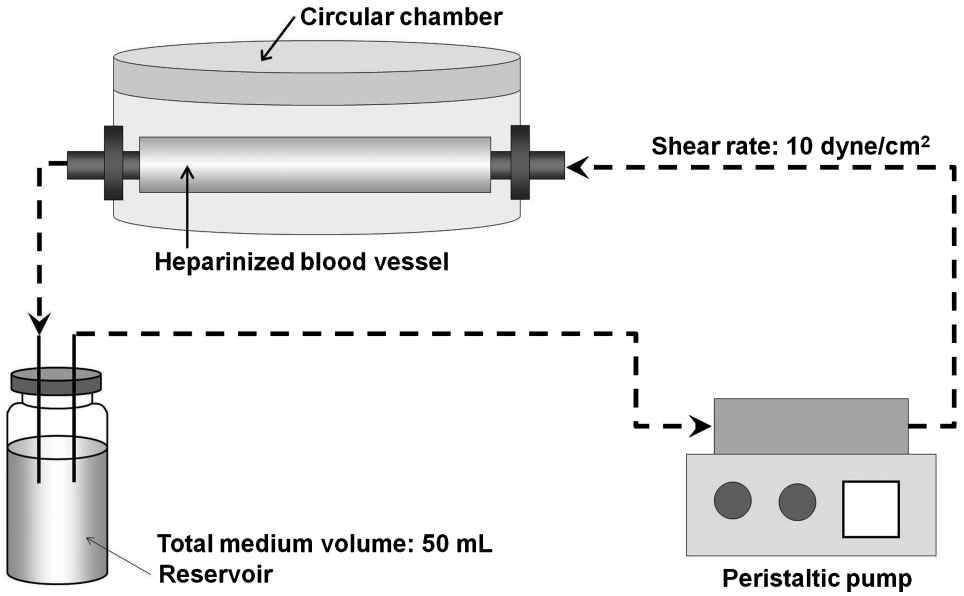


Fig. 4. Flow system for heparin release kinetic study.

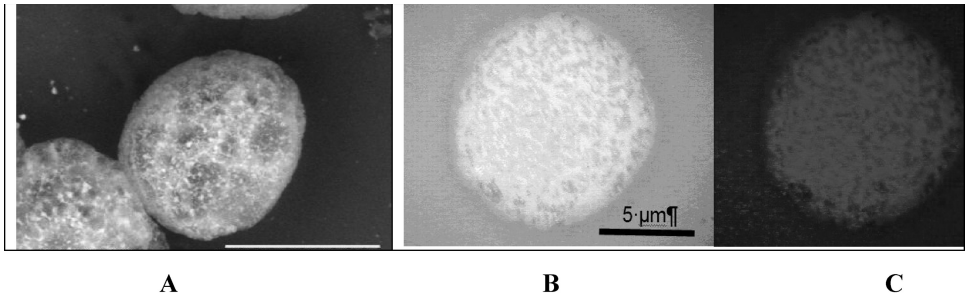
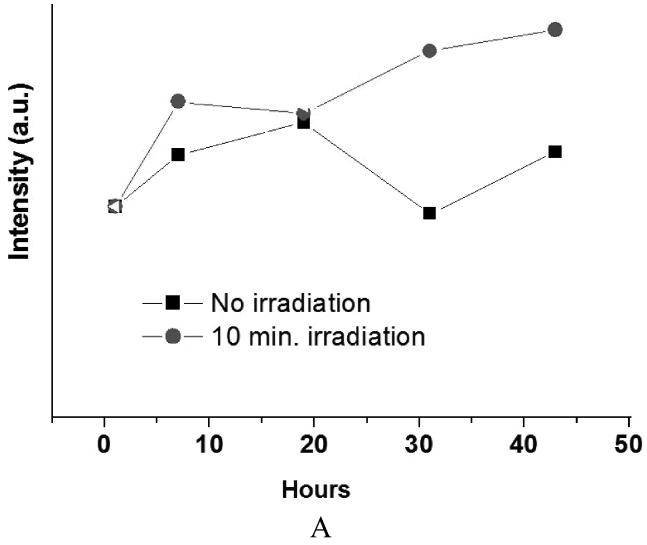


Fig. 5. A. SEM image of microcapsules. Scale bar is 20 μm; B. Fluorescence image of a single microsphere containing green (535 $\pm$ 10 nm emission) QDs at 20°C illuminated with a halogen lamp, Excitation Filter: 450 nm short pass filter. Emission: 450 nm long pass filter; C. False color image after post-processing by stripping red and blue channels from image and by increasing the contrast. The green area indicates areas of high fluorescent emission from the QDs.



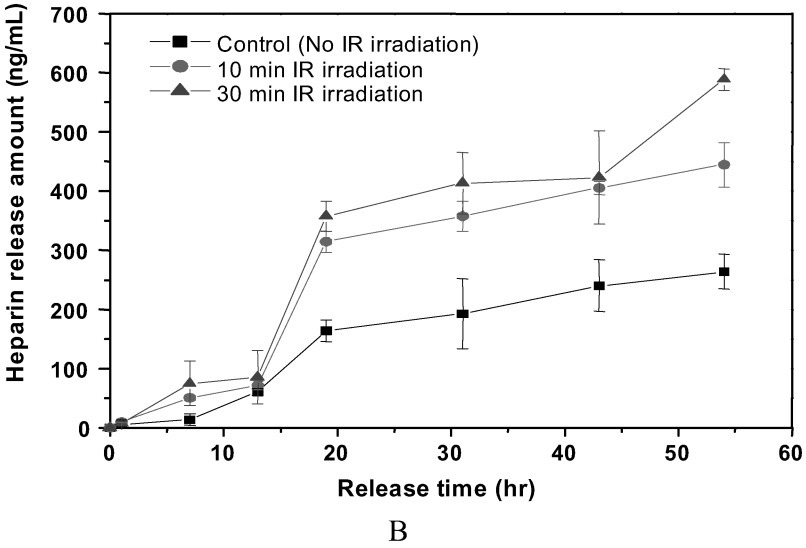


Fig. 6. Microspheres containing NIR QD-heparin show increased heparin release upon irradiation compared with control. Heparin release was analyzed by (A) optical and (B) biochemical analysis.

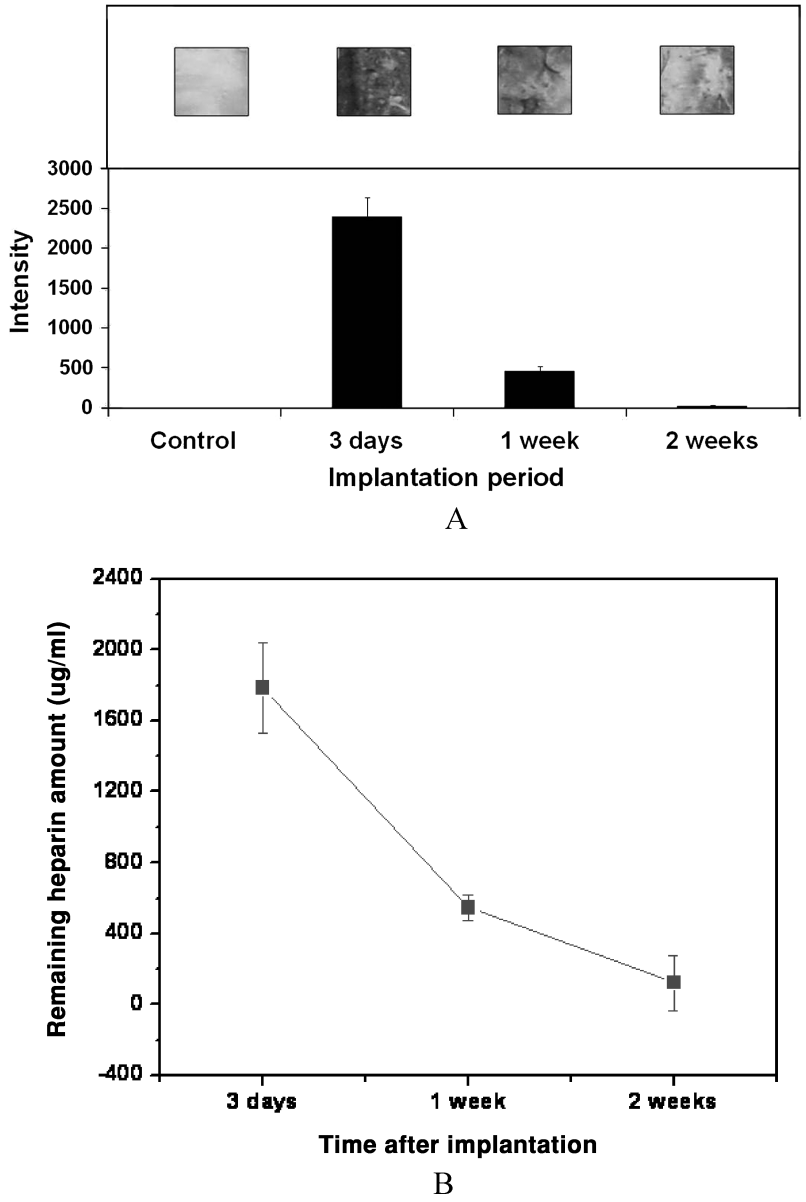


Fig. 7. Determination of remaining heparin in functionalized blood vessel with QD implanted in mice by (A) toluidine blue staining and (B) biochemical assay.

2.10.1. *In vivo release of heparin*

Quantum dot-heparin incorporated decellularizd vascular grafts were implanted in three mice for two weeks. The implanted vascular grafts were retrieved at determined times from animals and were frozen, pulverized in the presence of liquid nitrogen with a blender, and homogenized. Then 20% propylene glycol in the Q1 buffer (50 mM sodium acetate, 300 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethanesulphonylflouride (PMSF), pH 6.0) was used for the extraction of heparin without the use of detergents, yet retaining the biological activity. Heparins were extracted for 18 h at 4 degree C (in excess of 100 v/w) in Q1 buffer. The extracts were centrifuged for 30 min at 40,000 g and the glycosaminoglycans (GAGs) in the supernatant were quantitatively analyzed using a kit Rotachrom (Diagnostica Stago Inc).

The remaining heparin in the decellularized blood vessel was also assessed with toluidine blue staining. Briefly, after incubation in PBS for 5 min, the heparinized grafts were staining during 1 min using a 0.1% toluidine blue in DI water. The grafts were washed in PBS (three times for 5 min) and dehydrated in a graded series of ethanol (50, 60, 70, 80, 90, and 100%) for 5 min each.

3 Results and Discussion

3.1 Microencapsulation

Microcapsules were observed under SEM. The average size of microcapsules was $20 \pm 10\ \mu$m. Figure 5 shows a fluorescence image of a single microsphere which contains heparin conjugated to a green fluorescent QD. The microcapsules can be sterilized with gamma irradiation with no ill effects. Our data show that it is possible to bind heparin to QDs and encapsulate the QD-heparin in a biodegradable polymer.

3.2 *In vitro* Release of Heparin and Burst Release by IR Iirradiation in Polymer Microsphere

To assess the effectiveness of QDs for burst release of heparin, we analyzed the kinetics of heparin release following irradiation. Irradiated microspheres showed increased luminescence over time indicating a "burst release" (Fig. 6A). Quantification of heparin concentration showed burst release of heparin over 20 hr after irradiation. Subsequently the heparin was released at a constant slow rate (Fig. 6B). These results indicate that irradiation can be used to initiate a burst release of heparin from NIR QD-heparin containing microcapsules.

3.3 Heparin Release from Decellularized Blood Vessel in Flow System

The release kinetic profile demonstrated that the sustained release of heparin from functionalized vascular graft. Approximately 50∼60% of heparin was released in 10 days *in vitro* (data not shown).

3.4 *In vivo* Release of Heparin from Decellularized Vascular Graft in Mouse

To assess the effectiveness of the heparin *in vivo* model, heparin retention in and release from the decellularized blood vessels functionalized with QD-heparin were evaluated after the explantation of the vascular scaffold from mice. The remaining heparin in the vascular graft, determined by toluidine blue staining, showed that most of the heparin is shown to have diffused out of the vessel two weeks after implantation (Fig. 7A). The heparin content was analyzed by a Rotachrome kit and the data confirms that very little heparin remains after two weeks (Fig. 7B). The data show that we successfully prolonged the activity of heparin in the scaffold beyond its normal 1–2 hour half-life. *In vivo* NIR irradiation to implants needs to be followed for evaluation of the safety and efficacy of QD in regulating the release of heparin.

3.5 Host Response to QD-Heparin Vascular Scaffold in Mouse

The inflammatory response of QDs should be addressed for clinical applications. From the histological analysis of the explanted vascular scaffolds from mice, there was no evidence of inflammation or tissue encapsulation. The data indicate that conjugated heparin had only a minimal inflammatory response.

4 Conclusions

This paper showed a new approach in the field of controlled drug delivery systems using a novel quantum dot and heparin. We developed a system of microencapsulated drug which is conjugated to NIR absorbing QDs and evaluated the feasibility of burst release of drug from microcapsules by external irradiation. We have shown the feasibility of conjugating heparin to QDs and subsequent encapsulation. Burst release of heparin from microcapsules was achieved by irradiation.

These results suggest that QD-heparin microcapsules may be used for controlled release of heparin for various clinical applications especially for vascular graft engineering.

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References

1. Langer, R. (1998) *Nature,* 30(392), 5–10.
2. Peer, D., Karp, J.M., Hong, S., Farokhzad, O.C., Margalit, R. and Langer, R. (2007) *Nat. Nanotechnol.*, 2(12), 751–760.
3. Creque, H., Langer, R. and Folkman, J. (1980) *Diabetes,* 29, 37.
4. Sawicki J.A., Anderson D.G. and Langer R. (2008) *Adv. Exp. Med. Biol.,* 622, 209–219.
5. Finkelstein, A., McClean, D. and Kar S. (2003) *Circulation*, 107, 777–784.
6. Salvador-Morales C., Zhang, L., Langer, R. and Farokhzad, O.C. (2009) *Biomaterials,* 30(12), 2231–2240.
7. Finkelstein, A., McClean, D. and Kar, S. (2003) *Circulation*, 107, 777–784.
8. Lettino, M. and Toschi, V. (2004) *Curr. Med. Chem. Cardiovasc Hematol. Agents,* 3, 267–275.
9. Edelman, E.R., Nathan, A., Katada, M., Gates, J. and Karnovsky, M.J.(2000) *Biomaterials,* 21(22), 2279–2286.
10. Edelman, E., Kost, J., Bobeck, H. and Langer, R. (1986) *J. Biomed. Mater. Res.,* 19, 67–71.
11. Miyazaki, S., Yokouchi, C. and Takada, M. (1988) *J. Pharm. Pharmacol.*, 40, 716–717.

12. Saslawski, O., Weigarten, C., Benoit, J.P. and Couvreur P. (1988) *Life Sci.*, 42, 1521–1525.
13. Kost, J., Leong, K. and Langer, R. (1989) *Proc. Natl. Acad. Sci.*, 86, 7663–7666.
14. Levy, D., Kost, J., Meshulam, Y. and Langer, R. (1989) *J. Clin. Invest.,* 83, 2074–2078.
15. Cheng, J., Teply, B.A., Jeong, S.Y., Yim, C.H., Ho D., Sherifi, I., Jon, S., Farokhzad, O.C., Khademhosseini, A. and Langer, R. (2006) *Pharm. Res*., 23(3), 5570–5564.
16. Shenoy, D., Little S., Langer, R. and Amiji M. (2005) *Mol Pharm* 2(5), 357–366.
17. Bagalkot V., Zhang L., Levy-Nissenbaum, E., Jon, S., Kantoff, P.W., Langer R. and Farokhzad, O.C. (2007) *Nano. Lett*., 7(10), 3065–3070.
18. McAlister, V. (2006) *Clin. Invest. Med.,* 29(6), 373–377.
19. Lanthier, S., Armstrong, D., Domi, T. and deVeber, G. (2005) *Neurology* 22(64), 660–663.
20. Gilbert, T.W., Sellaro, T.L. and Badylak, S.F. (2006) *Biomaterials*, 27(19), 3675–3683.
21. Chun, S.Y., Lim, G.J., Kwon, T.G., Kwak, E.K., Kim, B.W., Atala, A. and Yoo, J.J. (2007) *Biomaterials,* 28(29), 4251–4256.