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Injectable Oxygen Sensitive Chitosan Complex with High Oxygen Sensitivity and Stability to Oxidoreductants

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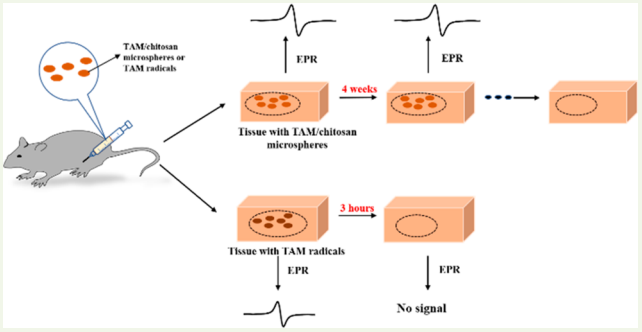
ABSTRACT: Various therapeutic approaches have been developed for vascularization of diseased tissues. To quickly and accurately evaluate efficacy of a therapy, reproducible monitoring of tissue oxygen concentration changes at the same tissue location by a minimally invasive or noninvasive spectroscopic approach represents a compelling strategy. Yet this cannot be achieved by any clinically available approaches. Magnetic-resonance-based method, electron paramagnetic resonance (EPR), has the potential to accomplish the goal. However, the existing small molecule EPR probes cannot maintain a consistent concentration at the same tissue location during vascularization period to acquire a stable EPR signal, and they cannot be implanted and/or retrieved by a minimally invasive approach such as injection. Herein, a polymeric, injectable, and degradable EPR probe was developed. The probe was based on the complex of chitosan and tetrathiatriarylmethyl (TAM) radical. The probe had high oxygen sensitivity and reproducibility. It was more stable than free TAM radical when exposed to oxidoreductants that commonly appear in diseased tissues. The probe maintained EPR signal intensity for 4 weeks in vitro and in vivo without changing oxygen sensitivity. It also exhibited excellent biocompatibility. The developed TAM/chitosan complex may be used for long-term detection of tissue oxygen content during therapies.



KEYWORDS: *chitosan, tetrathiatriarylmethyl radial, oxygen sensitivity, tissue oxygen measurement, electron paramagnetic resonance*

INTRODUCTION

Vascularization therapy is often used for diseased tissues with inferior blood perfusion. The purpose is to restore blood supply. To quickly and accurately assess therapeutic effect, noninvasive monitoring of tissue oxygen concentration changes at the same tissue location during the therapy period represents an attractive strategy.[1] Vascularization therapy generally takes more than 4 weeks. Tissue oxygen concentration gradually increases during the therapy.[2,3] Therefore, long-term (≥4 weeks) detection of oxygen concentration is necessary. Clinically available measurement approaches include transcutaneous oximetry,[4] pulse oximetry,[5] fiber optic oxygen probes,[6] and polarographic needle oxygen electrode.[7] However, these approaches either cannot long-term measure tissue oxygen or are invasive.[8] Magnetic resonance method EPR oximetry has potential for the noninvasive and long-term monitoring of tissue oxygen concentration.[1,9] EPR oximetry is based on the electron−electron spin−spin interaction between paramagnetic oxygen and exogenous spin probes.[10] It leads to a linear dependence of the peak-to-peak line width of spin probes and the oxygen concentration. The distinct advantage of EPR oximetry is its ability to measure tissue oxygen concentration while not consuming oxygen.[11−17] It also provides absolute values of oxygen concentration.[18,19]

Two types of EPR probes have been used for tissue oxygen concentration measurement, including water insoluble paramagnetic particles like paramagnetic lithium phthalocyanine and lithium octa-*n*-butoxynaphthalocyanine, and water-soluble probes such as triarylmethyl (TAM) and nitroxyl radicals. Current EPR probes can only be used for short-term oxygen measurement and cannot be used for the measurement at the same tissue location for extended periods. Those water-soluble probes cannot immobilize in tissues. Their concentration gradually decreases as the probes disperse in the tissues, leading to a progressive decay of EPR signal.[18,19] Repeated injection of EPR probes into tissues may have toxic effects.[8] The water insoluble EPR probes are not degradable. Therefore, even if they have excellent oxygen sensitivity, they cannot be readily cleared from the body, causing toxicity concerns.[8,14,18−23] Encapsulation of these EPR probes into highly oxygen permeable polymers such as PDMS may allow the probes to remain at the same tissue location. However, the resulting devices are not injectable and degradable. Invasive surgical procedures are required to implant and retrieve them.[18,19]

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Among the different EPR probes, TAM radical is particularly attractive for in vivo applications due to sharp singlet signal and narrow line width at anoxia and high oxygen sensitivity. To increase TAM radical retention time in tissues, it has been encapsulated into nondegradable poly(vinyl acetate) nanocapsules[24] or Pluronic F-127 hydrogel.[25] These approaches extended TAM radical retention time in vivo. Yet the retention time is still shorter than 5 h as the small molecular weight TAM radical can readily be released from the polymers. One of disadvantages of the encapsulation approach is that it decreased oxygen sensitivity due to the lower oxygen permeability of polymers.[25] In addition, the polymers used are nondegradable, which may lead to chronic inflammation in vivo. Conjugation of dendritic poly(ethylene glycol) to TAM radical has also been explored to increase retention time.[26] The half-life can be increased to ≥10 h. However, longer retention time is desired for long-term monitoring of oxygen content in tissues especially during vascularization process that takes several weeks.[27−29] Thus, there is a critical need for TAM based probes that can be retained in tissues for multiple weeks without changing high oxygen sensitivity.

To achieve the above goals, TAM/chitosan complex with in vivo retention time of at least 4 weeks was fabricated. The in vitro and ex vivo oxygen responses of the complex before and after degradation, biocompatibility, and stability to oxidoreductants were investigated.

MATERIALS AND METHODS

Materials. Chitosan, hydrogen peroxide (30% aqueous solution), ascorbic acid (Asc), iron(III) chloride hexahydrate, polycaprolactone (PCL), and acetone were purchased from Sigma-Aldrich. Nitrilotriacetic acid (NTA) was obtained from Fisher Scientific. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from Oakwood Chemical. All materials were used as received without further purification. Tris(8-carboxyl-2,2,6,6-tetramethylbenzo[1,2-*d*;4,5-*d*2]-bis[1,3]dithiol-4-yl)methyl TAM radical was synthesized following established protocol.[15]

Fabrication of TAM/Chitosan Complex. Chitosan and TAM radical were dissolved in HFIP to form solutions with concentration of 20 mM and 2 mM, respectively. The TAM/chitosan complex was obtained by slowly adding TAM radical solution into chitosan solution at the molar ratio of 1:180 (mass ratio of TAM and chitosan 1:24) under stirring overnight. The mixture was cast in a glass Petri dish. The film of TAM/chitosan complex was formed after evaporation of HFIP.

Water content of the TAM/chitosan complex film was tested after incubation in 37 °C DI water for 24 h. Weight of the wet film (w_1) and the lyophilized film (w_2) was measured, respectively. The water content was calculated as

$$\text{water content } (\%) = \frac{w_1 - w_2}{w_2} \times 100 \quad (1)$$

To test TAM/chitosan complex property change during incubation in aqueous condition for extend period, the film was immersed in 37 °C phosphate buffer saline (PBS, pH 7.4). The incubation was conducted for 4 weeks following our previous study.[30] Chitosan was used as control. At each time point, four samples for each group were taken out. Weight of the dry samples before and after incubation was recorded as w_3 and w_4, respectively. The weight remaining was determined as

$$\text{weight remaining } (\%) = \frac{w_4}{w_3} \times 100 \quad (2)$$

Oxygen Response of TAM/Chitosan Complex by EPR Measurement. EPR spectra were recorded by using Bruker EMX plus X-band resonator (9.8 GHz). The data were acquired by Xenon software. During the tests, modulation frequency, microwave power, modulation amplitude, magnetic field scan time, central field value, scan width, and number of scans accumulated for each spectrum were set as 100 kHz, 0.63 mW, 0.1 G, 60 s, 3364 G, 10 G, and 3 scans for each spectrum, respectively. To determine the effect of oxygen concentration on EPR spectra, the circular film (20 mg, 0.2 mm in thickness, and 20 mm in diameter) was immersed in PBS overnight and loaded into a gas permeable EPR tube. Gas with certain oxygen concentration was flushed into the tube to reach equilibrium. After flushing, EPR spectrum was recorded, and the peak-to-peak line width was automatically determined by Xenon software. Gases with different oxygen concentrations (0%, 10%, 20%, and 50%) were used. To evaluate reversibility of the samples during EPR measurements, pure nitrogen was flowed across samples after EPR line width reached the equilibrium in air. The change in EPR line width was recorded every minute. After the line width became stable, air was switched back and the line width was recorded every minute until EPR line width came to equilibrium.

Stability of TAM/Chitosan Complex When Exposed to Oxidoreductants. To test stability of the TAM/chitosan complex when exposed to oxidoreductants, H_2O_2 solution (1 mM), Asc solution (1 mM), and hydroxyl radicals (HO•) were used following a previously established method.[26] To generate HO•, $FeCl_3$ solution was slowly added into the NTA solution (molar ratio of 1:2) under vigorous stirring. Then, the mixture was neutralized to pH 7.4 by 0.1 M NaOH solution. HO· was released by mixing 0.1 mM Fe(III)-NTA solution with 1 mM H_2O_2 solution. EPR signals were measured after applying different oxidoreductants to TAM/chitosan complex for 30 min. The stability of TAM/chitosan complex was determined by the percentage of remaining TAM radical calculated by comparing the double integral of the EPR signal before and after exposure to the oxidoreductants.

Fabrication of TAM/Chitosan Complex Microspheres. The TAM/chitosan complex solution in HFIP was prepared following the aforementioned procedure. The solution was electrosprayed under a voltage of +15 kV to an aluminum pan charged with a voltage of −10 kV. The infusion rate of the TAM/chitosan complex solution was 2 mL/h. To obtain better dispersed microspheres, PCL/acetone solution with a concentration of 15 wt % was electrospun into the pan. The infusion rate was 10 mL/h. After fabrication, PCL was washed away by using acetone. The TAM/chitosan complex microspheres were then vacuum-dried and stored at −20 °C.

Implantation of TAM/Chitosan Complex and ex Vivo Evaluation of Its Oxygen Response. All animal experiments were conducted in accordance with the National Institutes of Health Guide for handling laboratory animals and the protocol approved by the Institutional Animal Care and Use Committee of The Ohio State University. TAM/chitosan complex microspheres were suspended in PBS at the concentration of 20 mg/mL. Male mice were anesthetized by using isoflurane. 200 μL microsphere suspension was injected into the thigh muscles by a 22G needle. The control was 200 μL of TAM radical in PBS with concentration of 20 mg/mL. The tissues in the injection area were harvested after 3 h and 4 weeks, respectively. The tissues were loaded into EPR tubes. EPR line widths were measured at different oxygen concentrations by using Bruker EMX plus X-band resonator (9.8 GHz).

Histology and Immunohistochemistry of Explanted Tissues. Muscle tissues were harvested after 2 weeks of implantation and fixed using 4% paraformaldehyde. The tissues were embedded in paraffin and sectioned into 4 μm slices. Hematoxylin and eosin (H&E) staining were performed. For the immunohistochemistry study, the slices were deparaffinized with xylenel and rehydrated with ethanol. Antibody against F4/80 was applied after blocking the slices with goat-serum for 1 h at room temperature. After incubation at 4 °C overnight, secondary antibody Alexa Fluor 488 was conjugated with the primary antibody. The images were taken by a confocal laser scanning microscope with excitation wavelengths of 488 and 633 nm.

Statistical Analysis. Data were expressed as the mean ± standard deviation. One-way ANOVA was applied for data analysis. Statistical significance was defined as $p < 0.05$.

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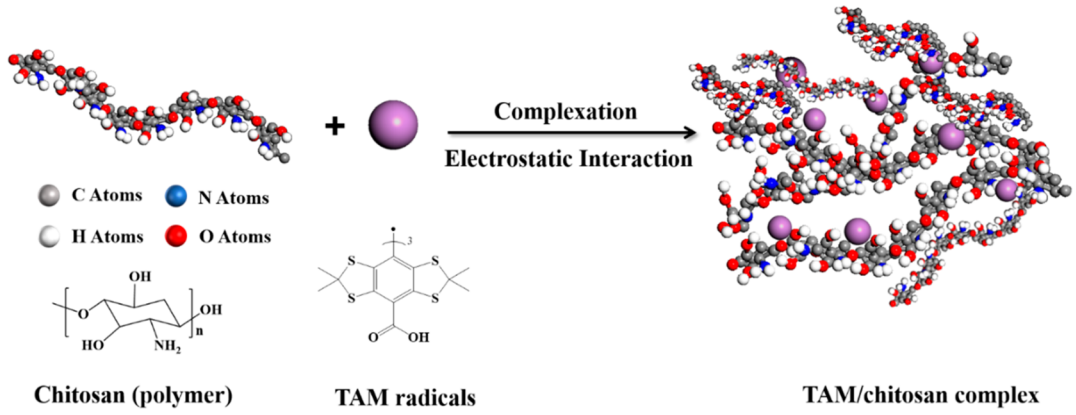

Figure 1. Complexation of TAM radical with chitosan. The molar ratio of chitosan and TAM is 180:1.

RESULTS AND DISCUSSION

TAM/Chitosan Complex Fabrication and Physical Properties. Chitosan has $-NH_2$ groups, suitable for complexation with $-COOH$ groups in TAM radical to form a stable complex (Figure 1).[31−33] The complex was water insoluble, thus enabling increase of TAM radical retention time in tissues compared to free TAM radical that readily diffuses in tissues to lose EPR signal. Chitosan degrades relatively slowly, allowing the complex to be used for long-term oxygen detection. In addition, chitosan has good oxygen permeability in a liquid environment[34] and excellent biocompatibility. During complexation, hexafluoroisopropanol (HFIP) was used as a solvent due to its high polarity and fast evaporation rate. The TAM/chitosan complex was relatively hydrophilic with water content of 43.4 ± 4.2%.

Oxygen Response of TAM/Chitosan Complex. After complexation of chitosan and TAM radical, the EPR spectrum showed a slight change for the background peaks, possibly due to the existence of chitosan. However, the TAM/chitosan complex still exhibited sharp singlet EPR spectrum under anoxic condition (inserted in Figure 2). The peak-to-peak line width (136 mG) was similar to that of the free TAM radical (135 mG). To evaluate oxygen response of the TAM/chitosan complex, line width at different oxygen concentration was measured. The complex demonstrated linear response of line width vs oxygen concentration where line width was increased from 136 mG at anoxia to 330 mG at 50% O_2. This increase was larger than that of the free TAM radical (135 mG at anoxia and 306 mG at 50% O_2). The TAM/chitosan complex possessed oxygen sensitivity of 0.51 mG/mmHg, slightly greater than that of the free TAM radical (0.45 mG/mmHg). These results demonstrate that complexation of TAM radical with chitosan through electrostatic interaction between $-NH_2$ and $-COOH$ groups largely preserved properties of the TAM radical. The results also suggest that the protons in chitosan did not have apparent influence on TAM radical. This is advantageous over chemical conjugation of TAM radical. A previous study found that using amide group to conjugate TAM radical with low molecular weight PEG not only broadened line width at anoxia but also decreased oxygen sensitivity, attributing to hyperfine splittings from the N and H nuclei of the amide group directly linked to the aromatic rings.[21] In this report, the peak-to-peak line widths of free TAM radical in anoxia and normoxia are larger than those in the previous report.[26] This possibly resulted from different settings used for EPR testings. Nevertheless, the linear relationship between line width and oxygen content was preserved in current study. This allows determination of oxygen content based on EPR line width.

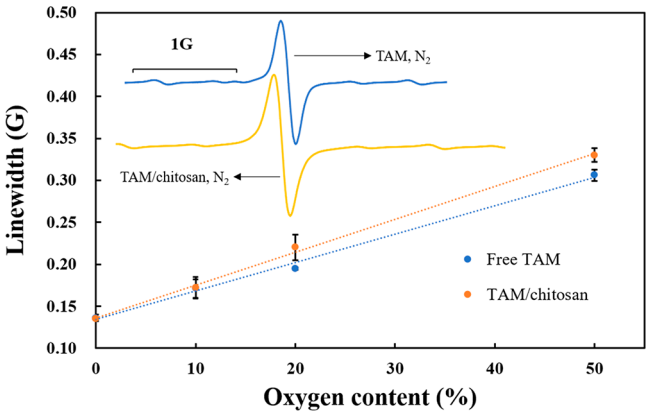

Figure 2. Linear relationship of oxygen content and EPR line width for free TAM radical and TAM/chitosan complex. Inserted EPR signals are for TAM and TAM/chitosan complex under nitrogen. No significant chemical shift, signal intensity, and peak-to-peak line width of EPR spectra were observed after the complexation of TAM radicals with chitosan.

For oxygen-sensing EPR probes, the rate of response to oxygen content change, and reversibility are two important properties. To determine whether complexation with chitosan has effect on these properties, the response rates and reversibility of free TAM radical and TAM/chitosan complex were measured in PBS. When the atmosphere was quickly switched from 20% oxygen to pure nitrogen, the line width of both free TAM radical and TAM/chitosan complex reached equilibrium state in 3 min (Figure 3). The response rate of free TAM radical is consistent with a previous study.[26] To evaluate reversibility, the atmosphere was rapidly changed from pure nitrogen to 20% oxygen. Both free TAM radical and TAM/chitosan complex reached stable line width in 2.5 min (Figure 3). These results demonstrate that the TAM/chitosan complex possessed the same response rate of oxygen concentration change and reversibility as free TAM radical.

Oxygen Response of TAM/Chitosan Complex during in Vitro Incubation in PBS. To determine whether TAM/chitosan complex remained reproducibility for oxygen detection during the in vitro incubation in aqueous condition, the samples were immersed in PBS at 37 °C for 2 and 4 weeks,

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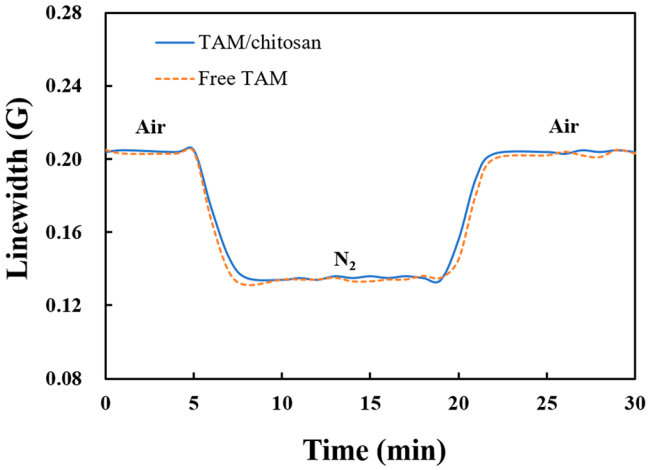

Figure 3. Reversibility of free TAM radical and TAM/chitosan complex determined by the change of EPR line widths as a function of time during gas switching.

respectively. The relationship of line width vs oxygen concentration was then measured. At both time points, the EPR line widths linearly increased with oxygen concentration (Figure 4a). The line width under anoxia was similar to that before incubation. These results demonstrate that the long-term exposure to aqueous condition did not change proton environment in TAM radical in the TAM/chitosan complex. At 2 and 4 weeks, the sample oxygen sensitivity remained the same (0.45 mG/mmHg). This oxygen sensitivity was also the same as that at week 0 (0.45 mG/mmHg), indicating that incubation did not change oxygen sensitivity. These results also demonstrate that the TAM radical in the TAM/chitosan complex was stable. To elucidate the relationship between EPR properties and weight loss, weight change during the incubation was quantified. No significant weight loss was found during the 4 week period as weight remaining of each week was similar to that of week 0 ($p > 0.4$, weight remaining of each week vs week 0) (Figure 4b). This is consistent with EPR results.

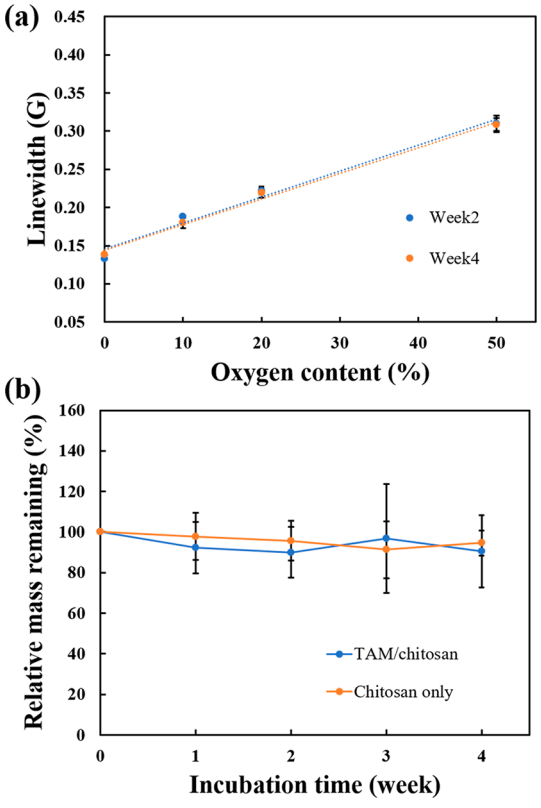

Figure 4. (a) Relationship of oxygen content and EPR line width for TAM/chitosan complex after incubation in 37 °C PBS for 2 and 4 weeks. (b) Weight remaining of TAM/chitosan complex and chitosan after incubation in 37 °C PBS for 4 weeks.

Stability of TAM/Chitosan Complex When Exposed to Oxidoreductants. Vascularization therapy is typically for ischemic tissues. Yet these tissues often contain oxidoreductants such as H_2O_2, Asc, and HO·. These oxidoreductants may quickly interact with EPR probes and impact their stability.[26] When free TAM radical was exposed to the oxidoreductants for 30 min, the EPR signal intensity was quenched to ~90% in H_2O_2, ~94% in Asc, and ~60% in HO· (Figure 5). These

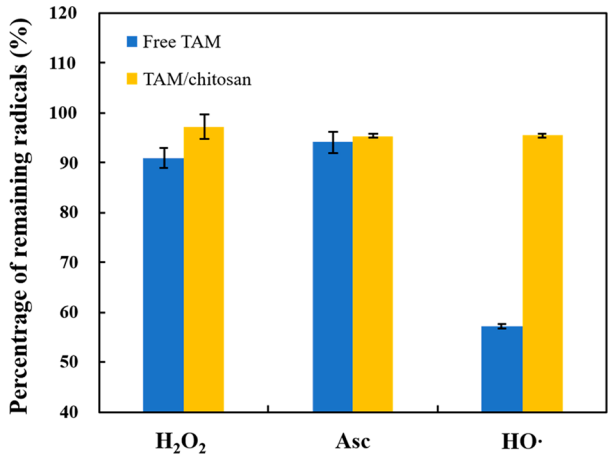

Figure 5. Stability of TAM/chitosan complex toward different oxidoreductants after incubation for 30 min. The concentration of H_2O_2 and Asc was controlled at 1 mM.

results are consistent with those reported previously.[26] The reducing agent Asc induced the reduction of TAM radical while oxidizing agents H_2O_2 catalyzed the oxidization of TAM radical, leading to a decrease in stability.[35,36] The underlying mechanism of HO· reduced TAM radical stability is not clear. The complexation of TAM radical with chitosan enhanced the stability of TAM radical in both H_2O_2 and HO· where the EPR signal intensity was significantly greater than that of the free TAM ($p < 0.05$). The complexation also slightly increased TAM radical stability when exposed to Asc. It is possible that the long, coiled, and intertwined chitosan chains effectively prevented TAM radical from exposure to the oxidoreductants. Previous studies demonstrated that conjugation of dendritic structure to TAM radical improved their stability in oxidoreductants.[20,26] The complexation of TAM radical with chitosan chains represents a more convenient and efficient way to augment TAM radical stability to oxidoreductants.

Fabrication of Injectable TAM/Chitosan Microspheres and Oxygen Response of the Microspheres. To make the TAM/chitosan complex injectable so that it can be delivered into tissues using a minimally invasive injection approach, the complex was fabricated into microspheres. An electrospraying technique was used for the fabrication (Figure 6a). The morphology of microspheres was observed by SEM

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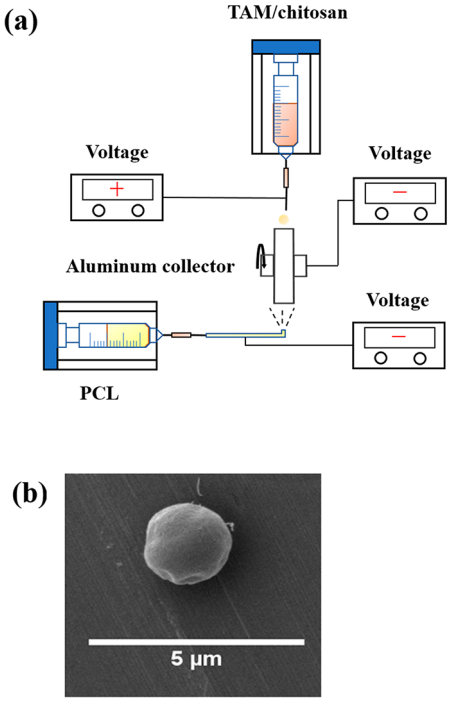

Figure 6. (a) Scheme of fabrication of TAM/chitosan complex by electrospraying. (b) SEM image of TAM/chitosan microsphere.

(Figure 6b). The microspheres assumed a diameter of 3.0 ± 1.1 μm (calculated from SEM image). They can be readily dispersed in PBS. To prepared 20 mg/mL suspension, an amount of 20 mg of microspheres was dispersed in 1 mL of PBS. The resulting suspension showed excellent injectability through 22G needles, typically used for muscle injection. The microspheres remained oxygen responsive. The line width of the microspheres suspended in PBS was proportional to the oxygen content, increasing from 127 mG at anoxia to 356 mG at 50% O_2. The oxygen sensitivity of the microspheres was 0.60 mG/mmHg (Figure 7). Compared to the TAM/chitosan

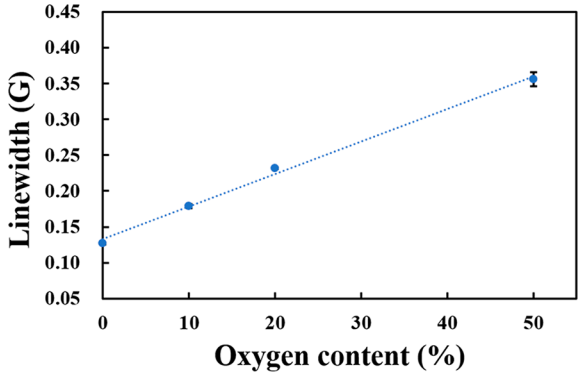

Figure 7. Relationship of oxygen content and EPR line width for TAM/chitosan microspheres. The concentration of TAM/chitosan microspheres was 20 mg/mL in PBS.

complex film, the microspheres exhibited lower line width in the anoxic condition and higher oxygen sensitivity. These results demonstrate that the fabricated TAM/chitosan microspheres can better detect oxygen than TAM/chitosan film. It is possible that the higher surface area-to-volume ratio of the microspheres facilitated oxygen to interact with TAM radical in the complex.

Oxygen Response of TAM/Chitosan Microspheres after Implantation. To determine the performance of microspheres after in vivo implantation, the microspheres were suspended in PBS and injected into murine thigh muscles. Free TAM radical dissolved in PBS was used as control. After 3 h and 4 weeks, the tissues were harvested, and EPR measurements were conducted ex vivo under different oxygen concentrations. For the free TAM radical injected into tissues for 3 h, the intensity of EPR signals under anoxia was greatly weakened, and the line width was largely increased to 2500 mG (Figure 8b). These results demonstrate that free

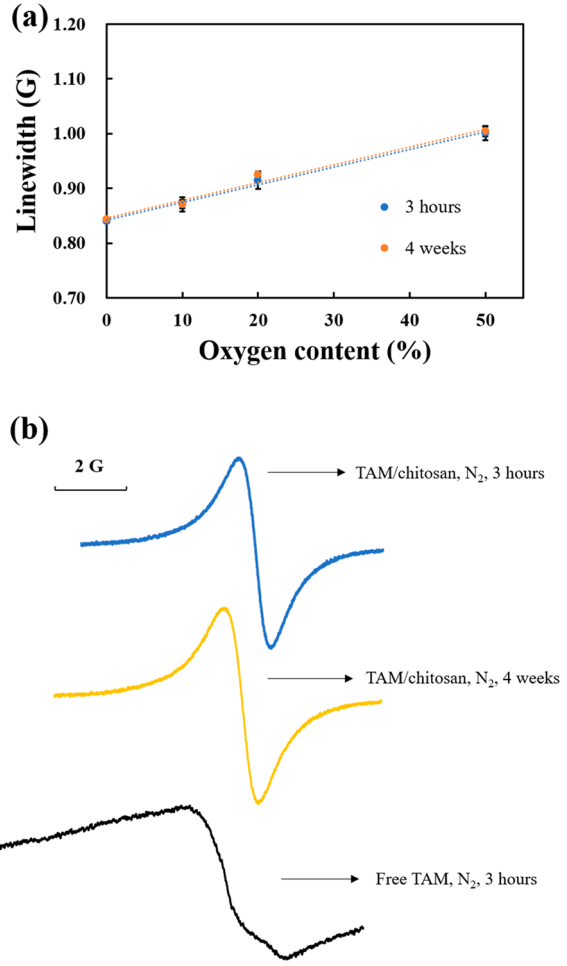

Figure 8. (a) EPR line width of TAM/chitosan microspheres as a function of oxygen content after implantation into mouse thigh muscle for 3 h and 4 weeks and (b) EPR signals under nitrogen for free TAM radical after 3 h of implantation and for TAM/chitosan microspheres after 3 h and 4 weeks of implantation. For EPR tests, modulation frequency, microwave power, and modulation amplitude were set as 100 kHz, 0.63 mW, and 0.1 G, respectively. The g value was 2 for all three EPR spectra.

TAM radical has low stability in tissues. This is consistent with previous reports where free TAM radical and its soluble derivatives have short half-lives in tissues.[26,15] It is likely due to binding with plasma proteins.[37] The complexation of TAM radical with chitosan largely increased its half-life. After 3 h of implantation, the TAM/chitosan microspheres under anoxia exhibited singlet and sharp EPR signals, and the line width

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(840 mG) was much narrower than that of the free TAM radical (Figure 8b). The increased half-life is attributed to chitosan. The long polymer chains limited plasma proteins to access complexed TAM radical.

After 4 weeks of implantation, similar EPR signals as for 3 h of implantation were found for the TAM/chitosan microspheres (Figure 8b). In addition, the line widths at anoxia remained similar (841 mG). At both 3 h and 4 weeks of implantation, TAM/chitosan demonstrated a linear function of line width and oxygen content (Figure 8a). The oxygen sensitivity was also remained similar (0.42 and 0.43 mG/mmHg at 3 h and 4 weeks, respectively). Chitosan is degradable. The degradation of chitosan may release TAM radical, leading to the decrease of signal intensity or compromised oxygen sensitivity. The results in the above studies demonstrate that the developed TAM/chitosan microspheres did not undergo substantial degradation in vivo for 4 weeks. Therefore, the TAM/chitosan microspheres may be able to detect tissue oxygen concentration for 4 weeks. In our future studies, we will inject these microspheres into ischemic tissues and real-time-monitor tissue oxygen concentration over a 4-week period.

Biocompatibility of the TAM/chitosan microspheres was examined after 2 weeks of implantation. The thigh muscles without injection were used as control. H&E staining (Figure 9a) and F4/80 staining (Figure 9b) were performed for explanted tissues. Both stainings did not show substantial difference between the normal muscle tissues and those injected with TAM/chitosan microspheres. These results demonstrate that the injected TAM/chitosan microspheres did not cause substantial inflammation.

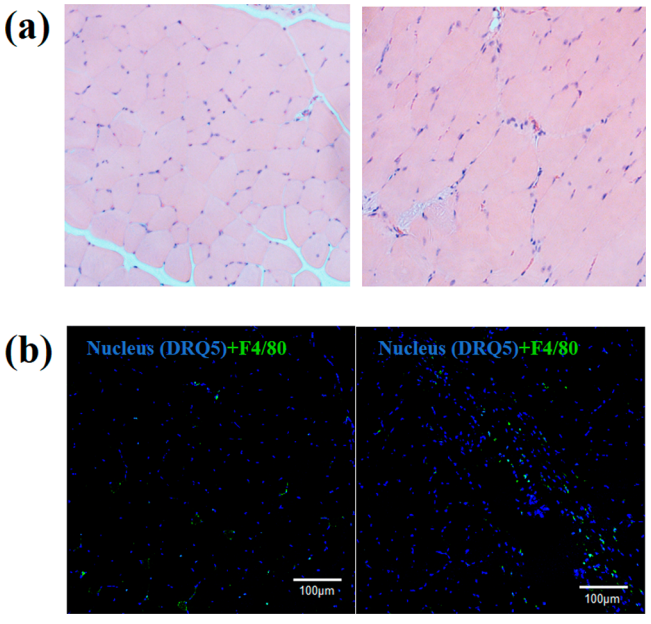

Figure 9. (a) H&E staining of muscle tissues without (left) and with (right) injection of TAM/chitosan microspheres and (b) F4/80 staining of tissues without (left) and with (right) injection of TAM/chitosan microspheres.

CONCLUSION

Degradable TAM/chitosan complex possessing high oxygen sensitivity and stability to oxidoreductants was developed. The TAM/chitosan microspheres can be implanted into tissues by a minimally invasive injection approach. The complexation of TAM radical with chitosan largely increased its half-life in vivo. The TAM/chitosan complex also exhibited excellent biocompatibility. The developed oxygen sensitive TAM/chitosan complex has the potential to be used to noninvasively, real-time, and long-term monitor tissue oxygen by EPR.

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Author Contributions

§C.L. and Z.H. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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