MOLECULAR DESIGN OF BIOLOGICALLY ACTIVE BIODEGRADABLE POLYMERS FOR BIOMEDICAL APPLICATIONS

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Abstract: The objective of this research is to synthesize synthetic biodegradable polymers that would have biological functions similar to nitric oxide. Polyglycolide (PGA) was the synthetic biodegradable polymer and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxy (Tempamine) was chosen as the source of nitroxyl radicals. Tempamine nitroxyl radicals were chemically incorporated into the carboxylic acid chain ends of PGA macromolecules via amide linkage. The kinetics of *in vitro* hydrolytic release of Tempamine nitroxyl radicals from the host PGA in buffered media at 37 °C was studied. Tempamine nitroxyl radicals were released into the media via cleavage of either ester linkages in the PGA segments or/and the amide linkage between Tempamine and the PGA segments. The duration of hydrolysis would determine the type of degradation products that were different in the segmental length of the PGA component. A preliminary *in vitro* cell culture study of this new generation of biologically active biodegradable polymers indicated that it was able to retard the proliferation of smooth muscle cells as pure nitric oxide does.

INTRODUCTION

All existing synthetic biodegradable and non-biodegradable polymeric biomaterials have been used for the reconstruction of injured, diseased or aged human tissues with different levels of

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success. Although these biomaterials are different in their chemical constituents, they have one common characteristic: they do not have any inherent biological functions which improve human body repair. They are not "biologically active" and play only a passive role in wound healing, tissue regeneration and tissue engineering. It would be ideal if these biomaterials could be made biologically "alive" and active by having some critical biological functions, such as the ability to modulate inflammatory reactions, to facilitate wound healing or to enhance host defenses against disease.

Many approaches have been experimented to design biologically active biodegradable biomaterials. These include the use of cytokines, such as growth factors, which impart biological functions on biomaterials. However, incorporation of a unique biochemical, nitric oxide (NO·), into synthetic biodegradable polymers to make them biologically "active" has received virtually no attention in the biomaterials community.

Nitric oxide is a very small but highly reactive and unstable free radical with expanding known biological functions. This molecule and its biological functions have recently become one of the most studied compounds in biochemistry and biology and the subject of several recently reviews (Refs. 1-11). NO· is extremely labile and short-lived (about 5 to 30 seconds).

NO·, and its nitroxyl radical derivatives, have been shown to play a very important role in a host of expanding biological functions, such as inflammation, neurotransmission, blood clotting, blood pressure, cardiovascular disorders, rheumatic and autoimmune diseases, antitumor activity with a high therapeutic index, antimicrobial property, sensitization or protection of cells and tissues against irradiation, oxidative stress, respiratory distress syndrome, and cytoprotective property in reperfusion injury, to name a few (Refs. 1-23). NO· acts both as an essential regulatory agent to normal physiological activities and as cytotoxic species in diseases and their treatments. Nathan et al. reported that nitric oxide is a potent antiviral compound against poxvirus and herpes simplex virus type-1, which causes cold sores in humans (Ref. 5). Levi et al. found that nitric oxide protects the human heart against low oxygen supply, a condition known as myocardial ischemia, by widening blood vessels so that more oxygen-rich blood reaches the heart (Ref. 7). Elliott et al. reported that a new NO· -releasing nonsteroidal anti-inflammatory drug has the benefits of accelerating gastric ulcer healing (Refs. 24,25). It is important to know, however, that excessive introduction of NO· into body may have adverse effects like microvascular leakage, tissue damage in cystic fibrosis, septic shock, B-cell destruction, and possible mutagenic risk, to name a few (Refs. 11,12,20,26-28).

In this paper, we report on a patented chemical synthesis for incorporating of nitroxyl radicals into synthetic biodegradable biomaterials, the kinetics of the release of nitroxyl radicals from the synthetic biodegradable biomaterials and a preliminary *in vitro* cell culture evaluation of the biological function of the newly designed biologically active biodegradable biomaterials. It is our belief that these new and biologically active biodegradable biomaterials could permit scientists and medical profession to actively repair injured, diseased or aged human tissues.

MATERIALS & METHODS

Synthesis of Nitroxyl Radical Incorporated Synthetic Biodegradable Polymers:

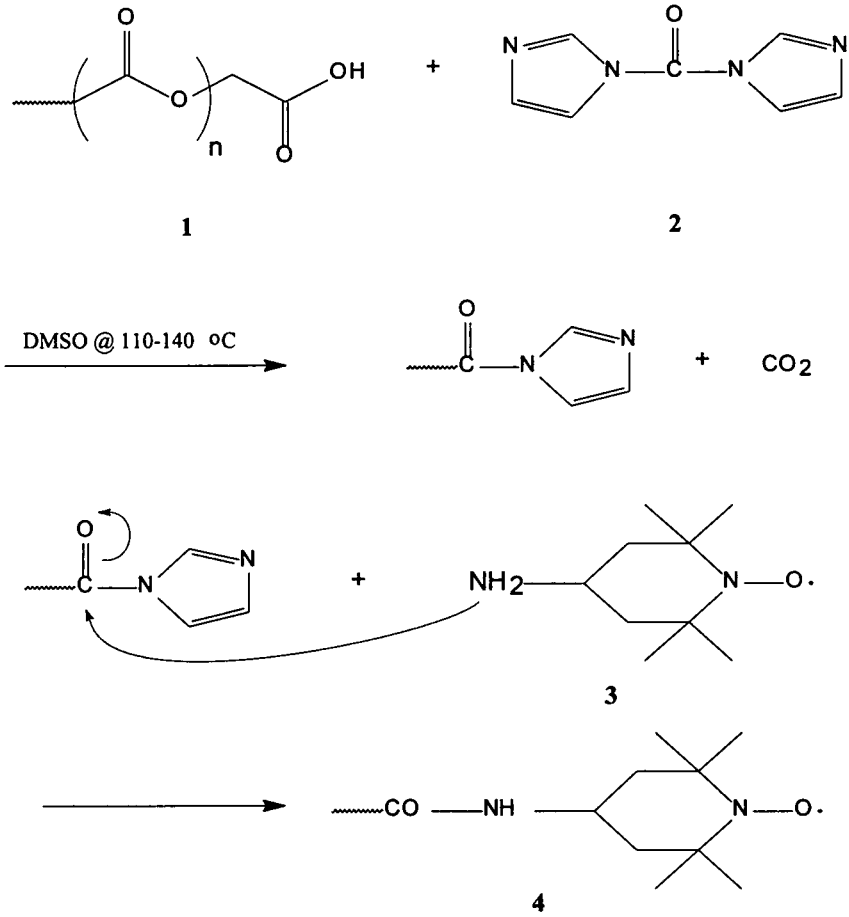
The following chemicals were used for synthesizing nitroxyl radical incorporated biodegradable polymers: Polyglycolide (PGA) as the biodegradable polymer (**1**), 4-amino-2,2,6,6-tetramethylpiperidine-1-oxy (TAM) as the source of nitroxyl radicals (**3**), N,N'-carbonyl diimidazole, and dimethylsulfoxide (DMSO) (**2**). The biodegradable PGA carrier has an inherent viscosity of about 1.3 at 25^{0}C and was obtained from Boehringer Co., (Ingelheim, Germany). TAM and N,N'-carbonyl diimidazole were purchased from Aldrich Chemical Co. The DMSO solvent was distilled in calcium hydride powder before use.

The PGA polymer (0.5g) was dissolved in 50 ml distilled DMSO at 110-140^{0}C. Then, N,N'-carbonyl diimidazole (8.1mg) was added. After 15 minutes, the TAM nitroxyl radicals (8.5mg) dissolved in DMSO were added slowly to the reaction mixture. The reaction mixture was then vigorously stirred for several hours while maintaining a temperature between 120-130^{0}C. The resulting solution mixture was then poured into a mixture of cold acetone and methanol (60/40,v/v) to precipitate the final product. These polymer products were recovered by filtering and purified by washing three times with cold methanol and then vacuum drying. **Scheme 1** illustrates the synthetic route of the TAM- incorporated PGA (TAM-PGA) (**4**). TAM nitroxyl radicals (**3**) were chemically bonded to PGA (**1**) carboxylic chain ends via amide linkages.

In Vitro Release of Nitroxyl Radicals from the Synthetic Biodegradable Polymers:

The TAM-PGA powers were placed into a micro centrifuge tube filled with 2ml 0.1M phosphate buffer solution of pH=7.44. The tube was then placed in an oven maintained at

37°C. The amount of TAM nitroxyl radicals released from the PGA upon *in vitro* hydrolysis was measured by electron paramagnetic resonance (EPR) as a function of hydrolysis time. The EPR spectra at X-band were obtained from a Bruker 200D SRC spectrometer operating at 9.6GHz, using 100KHz modulation. The EPR signal intensity (TAM nitroxyl radical concentration) was obtained by a double integration of the recorded first derivative signals and then calibrated with a standard cholesterol (CSL) spin solution. The pHs of the aliquots at different hydrolysis periods from were measured by using a micro glass electrode pH meter.



Scheme 1. Chemical synthesis of TAM nitroxyl radicals incorporated polyglycolide.

Preliminary *In Vitro* Cell Culture Study:

One of the well-known biological functions of nitric oxide is its ability to retard the proliferation of smooth muscle cells. In order to determine whether the newly synthesized TAM-PGA biomaterials provide a biological function similar to NO·, a preliminary *in vitro* cell culture study was conducted. Human intestinal smooth muscle cells (SMC) were purchased from ATCC (CRL-1692) and allowed to grow to confluence in tissue culture flasks in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. The confluent SMCs were trypsinized, resuspended in 30 mls DMEM, centrifugated, and after removal of supernatant and they were resuspended in 50 mls of the following media individually: DMEM+10% FBS (as medium control), PGA powders suspended in DMEM+10%FBS medium at 1 mg/ml (as substrate control), TAM-PGA powders suspended in DMEM+10%FBS medium at 1mg/ml, and TAM nitroxyl radicals in DMEM+10%FBS medium at 1, 10 & 100 μg/ml. The controls and testing samples were poured onto 25 cm^2 flasks with a SMC concentration of $1.25x10^5$ cells/flask. The flasks were incubated at 37°C in a 5% CO_2 incubator and their media were changed once per week over the entire period of 21 days. At the end of 2,4,7,14 and 21 days incubation, SMCs were trypsinized from the flask and counted by hemocytometer.

RESULTS & DISCUSSION

Due to the free radical characteristic of TAM nitroxyl radicals, the TAM incorporated PGA must exhibit an EPR spectrum that has the characteristic of nitroxyl radicals. **Figure 1** shows such an EPR spectrum of TAM-PGA. This EPR spectrum shows a broadening of linewidth when compared with the EPR spectrum of free nitroxyl radicals. Such a broadening of an EPR spectrum is due to the restriction of free radical motion from the viscous polymeric environment. The amount of TAM nitroxyl radicals that were incorporated into PGA was 7.455×10^{20} spin number/gram of PGA.

Because TAM nitroxyl radicals were incorporated only into the carboxylic chain ends of PGA macromolecules, their physical, thermal and mechanical properties are expected to be not significantly different from the parent PGA macromolecules. The lack of changes in these fundamental properties between TAM-PGA and parent PGA biomaterials may be advantageous

because the same processing conditions that have been used to fabricate PGA for a variety of clinical applications could also be used to fabricate the new TAM-PGA. In addition, the knowledge of the well-known biodegradation properties of PGA could be applied to estimate the release pattern of TAM nitroxyl radicals from PGA upon its biodegradation. The main difference between the parent and TAM-PGA, however, is their degradation products and their subsequent biological properties. The TAM-PGA was expected and demonstrated the release of TAM nitroxyl radicals which rendered biological activities to TAM-PGA that PGA did not have. Since the expected biological functions of the TAM-PGA must come from the nitroxyl radicals that are released into the surrounding environment upon hydrolytic degradation of PGA, the amount and the rate of release of TAM nitroxyl radicals should have a direct impact on the applicability of the newly synthesized TAM-PGA biomaterials to medicine. EPR spectroscopy was used to characterize the *in vitro* release pattern of TAM nitroxyl radicals from the TAM-PGA as a function of hydrolysis time..

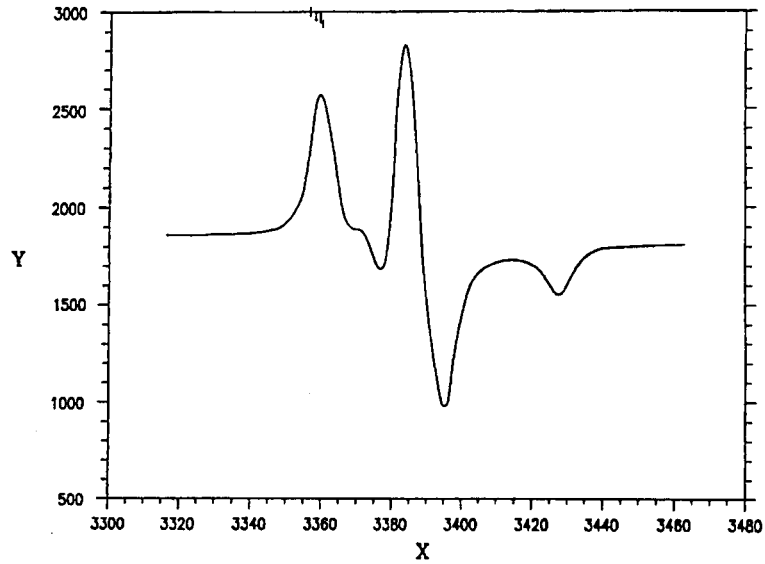


Figure 1. EPR spectra of TAM nitroxyl radicals incorporated polyglycolide (TAM-PGA).

Figure 2 illustrates such an *in vitro* release pattern of TAM radicals from PGA. The release of TAM nitroxyl radicals upon PGA hydrolysis followed a double exponential behavior in which significant amounts of nitroxyl radicals were released before 20 days followed by a gradua release thereafter.1

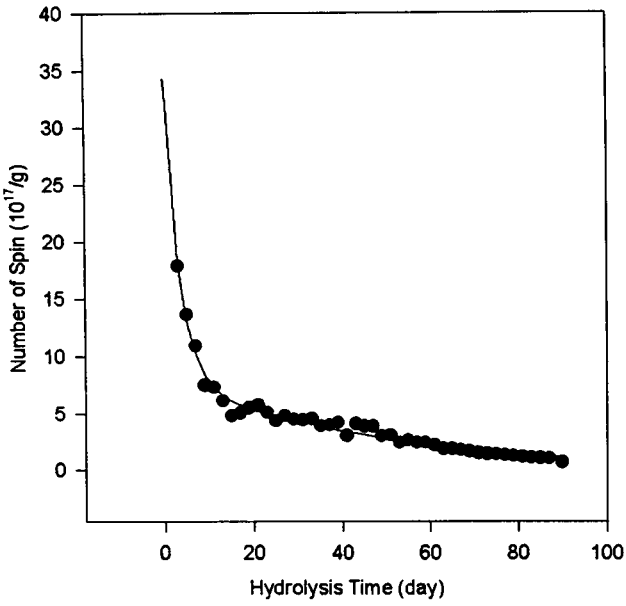


Figure 2. The kinetics of *in vitro* release of TAM nitroxyl radicals from TAM-PGA as a function of hydrolysis time at 37°C.

An examination of the pH of the degradation media indicated a similar double exponential reduction in pH. The pH of the medium was reduced from the initial 7.4 to 3.5 during the first 12 days of hydrolysis and there was very little reduction in pH thereafter. It was found that this reduction in pH would have a profound effect on the resulting TAM nitroxyl radicals in terms of their EPR signal intensity due to the possible chemical reduction of the radicals to

hydroxypiperidine via protonation in an acidic medium. Such a chemical reduction of TAM radicals to hydroxypiperidine would reduce their free radical characteristic and hence their EPR signal intensity.

The biological activity of TAM-PGA can best be illustrated by the level of retardation of SMC in cell culture. As shown in **Figure 3,** TAM-PGA showed profound retardation of the proliferation of SMC *in vitro*.

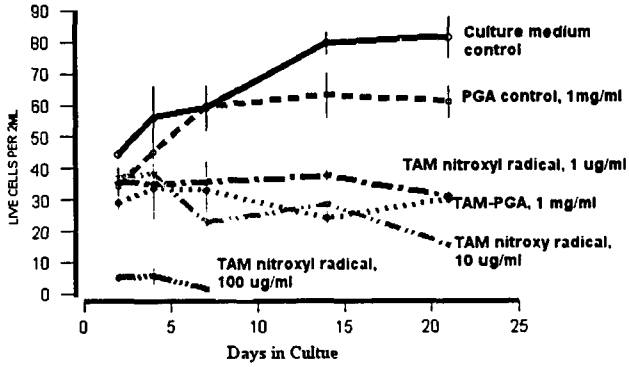


Figure 3. The effect of TAM-PGA and its controls on the proliferation of human smooth muscle cell culture.

There was virtually no change in the number of live SMCs over the entire period of cell culture in the culture medium having TAM-PGA, while the number of live SMCs in the culture medium of controls more than doubled (134% increase) during the same culture period. This level of SMC retardation of TAM-PGA biomaterial was found to be similar with free TAM nitroxyl radicals at 1 μg/ml. Figure 3 also shows that any higher concentrations of TAM nitroxyl radicals in the culture media (> 1 μg/ml) appear toxic to SMC as evident in the reduction in SMC population from the initial number (0 day), particularly at 100 μg/ml. It was a common concern that since the intermediate and final degradation products from the hydrolysis of TAM-PGA biomaterials are chemically different from pure nitric oxide, they might not have the same biological functions as simple nitric oxide. This concern appears not to be warranted because the retardation of SMC proliferation by TAM-PGA biomaterial

occurred as early as 3 days. Thus, it appears that both the free TAM and the TAM-PGA nitroxyl radicals would have the same biological function as pure nitric oxide. The long PGA chain segments that were attached to TAM nitroxyl radicals appear not to interfere with the biological functions of the nitric oxide portion of the TAM-PGA molecules. Since the level of retardation of SMC by TAM-PGA biomaterials was found to be similar to the pure TAM nitroxyl radicals at 1 μg/ml over the entire culture period, it appears that the amounts of TAM that were incorporated into PGA chain ends based on the stipulated chemical reaction conditions were adequate for this particular purpose.

Some examples of the potential use of this new generation of biologically active biodegradable polymers are the treatment of intimal hyperplasia after balloon angioplasty procedures, anticancer drugs, wound closure materials with improved healing and antimicrobial capability, and synthetic vascular grafts that would not reduce coagulation.

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REFERENCES

1. R.I. Zhdanov, "*Bioactive Spin Labels*", Springer-Verlag, Berlin (1992).
2. S.H. Snyder et al., *Sci. Amer.*, **5**, 68 (1992).
3. S.Moncada, et al., "*Nitric oxide: Physiology, Pathophysiology and Pharmacology*", in: *Pharmacol. Rev.*, Vol. 43, No.2, June (1991), pp.109.
4. D.S. Bredt, et al. *Nature* **351(6329)**, 714 (1991).
5. G.Karupiah et al. *Science*, **261(5127)**, 1445 (1993).
6. H. Esumi, et al. "U.S.- Japan Cooperative Cancer Research Program: Seminar on nitric oxide synthase and carcinogenesis (Williamsburg, VA, January 19-20, 1993)" *Cancer Res.*, **54(1)**, 297 (1994).
7. K.H. Park, L.E. Rubin, S.S. Gross and R. Levi, *Circulation Res.*, **71(4)**, 992 (1992).
8. V. Darley-Usmar et al. *FEBS Letters*, **369(2-3)**, 131 (1995).

9. S.M. Sagar et al. *Cancer Treatment Review*, **21(2),** 159 (1995).

10. C.J. Morris et al., "*Reactive Oxygen Species in Skin Inflammation*", in: *Handbook of Immunopharmacology: Immunopharmacology of Free Radical Species,*" Acad. Press, San Diego, CA, 1995, pp 113-125.

11. E. Moilamen et al. *Annals of Medicine*, **27(3),** 359 (1995).

12. R.H. Liu et al. *Mutation Research*, **339(2),** 73 (1995).

13. L.H. Pheng et al., *Inflammation*, **19(5),** 599 (1995).

14. A.R. Amin, et al., *Proceedings of Natl. Acad. Sci.*, **92(17),** 7926 (1995).

15. R.A. Star et al. *Proceedings of Natl. Acad. Sci.*, **92(17),** 8016 (1995).

16. M. Ikeda et al., *Japn. J. Pharmocol.* **68(2),** 183 (1995).

17. P.J. Barnes, *Annals of Medicine*, **27(3),** 389 (1995).

18. M.B. Ganz, et al. *Am. J. Physiol.*, **268(6 Part 2),** F1081 (1995).

19. J.A. Lovchik, et al., *Am. J. Respir. Cell & Mole. Biol.*, **13(1),** 116 (1995).

20. G.J. Dusting et al., *J. Vasc. Res.*, **32(3),** 143 (1995).

21. B. Halliwell, *Annals of the Rheumatic Dis.*, **54(6),** 505 (1995).

22. D.A. Wink, et al., *Arch. Biochem. & Biophys.*, **319(2),** 402 (1995).

23. E.M. Sotomayor, et al., *Inteln. J. Cancer,* **60(5),** 660 (1995).

24. S.N. Elliott, et al., *Gastroenterology*, **109(2),** 524 (1995).

25. J.L. Wallace, et al., *Europ. J. Pharmacol.*, **280(1),** 63 (1995).

26. H.H. Schmidt, et al. *Cell*, **78,** 919 (1994).

27. A.K. Nussler, et al. "*Inducible nitric oxide synthase: Its role in inflammation, sepsis, and shock,*" in: *Modulation of the Inflammatory Response in Severe Sepsis*, J.M.Telladoo et al. (Eds), *Progress in Surgery*, Vol.20, S. Karger AG, Basel, Wwitzerland, 1995, p.33.

28. C. Francoeur, et al., Inflammation, **19(5),** 587 (1995).