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Porous Bone Morphogenetic Protein-2 Microspheres: Polymer Binding and In Vitro Release

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ABSTRACT

This research compared the binding and release of recombinant human bone morphogenetic protein 2 (rhBMP-2) with a series of hydrophobic and hydrophilic poly-lactide-co-glycolide (PLGA) copolymers. Porous microspheres were produced via a double emulsion process. Binding and incorporation of protein were achieved by soaking microspheres in buffered protein solutions, filtering, and comparing protein concentration remaining to nonmicrosphere-containing samples. Protein release was determined by soaking bound microspheres in a physiological buffer and measuring protein concentration (by reversed-phase high-performance liquid chromatography) in solution over time. Normalized for specific surface area and paired by polymer molecular weight, microspheres made from hydrophilic 50:50 or 75:25 PLGA bound significantly more protein than microspheres made from the corresponding hydrophobic PLGA. Increased binding capacity correlated with higher polymer acid values. With certain polymers, rhBMP-2 adsorption was decreased or inhibited at high protein concentration, but protein loading could be enhanced by increasing the protein solution:PLGA (volume:mass) ratio or by repetitive soaking. Microspheres of various PLGAs released unbound protein in 3 days, whereas the subsequent bound protein release corresponded to mass loss. RhBMP-2 binding to PLGA was controlled by the acid value, protein concentration, and adsorption technique. The protein released in 2 phases; the first occurred over 3 days regardless of PLGA used and emanated from unbound, incorporated protein, while the second was controlled by mass loss and therefore was dependent on the polymer molecular weight. Overall, control of rhBMP-2 delivery is achievable by selection of PLGA microsphere carriers.

KEYWORDS: bone morphogenetic protein-2, PLGA microspheres, controlled delivery, protein delivery, in vitro.

INTRODUCTION

Bone Repair

More than 250 000 bone grafts are performed annually in the United States [1], and within the area of research broadly defined as tissue repair, the need for a bone graft substitute (BGS) continues to be driven by the huge demand [2]. Autogenous bone grafts, the bone tissue repair material of choice, have had modest success, but availability is limited.

Brekke and Toth [1] listed 7 general categories of requirements for osteoinductive BGS:

- biocompatibility
- gross architecture qualities
- osteoconduction
- chemotaxis
- angiogenesis and vascularization
- delivery and control of osteoinductive protein
- administrative issues

In the mid-1980s, the biotechnology industry began to make available the osteoinductive proteins responsible for bone growth. Brekke and Toth listed 17 specific device characteristics within the general category of "delivery and control of osteoinductive protein" for a BGS. Most important were "release of the osteoinductive agent at therapeutic levels and at the proper time" [3-5] and "release kinetics calibrated to local requirements" [3,6].

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Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a 32-kd homodimer currently being tested for its use in bone healing [7,8]. The protein's osteoinductive property of causing mesenchymal differentiation into chondrocytes, with subsequent calcification of the cartilaginous matrix, can be enhanced by prolonging its presence at the site of healing [9]. Clinical use of rhBMP-2 has been hampered by a lack of suitable delivery systems. Such systems should be capable of maintaining the protein in situ for sufficient time to interact with target cells, release the protein at effective concentrations during bone formation, cause no unnecessary tissue distress, and be resorbed [10].

A number of approaches have been used to encapsulate pharmacologically active agents into microspheres of polymers for sustained release [11,12]. Most are based on phase separation or emulsion evaporation or double-emulsion evaporation. The latter provides a route for efficient encapsulation of water-soluble, hydrophilic drugs [13].

Unfortunately, double-emulsion, or water-in-oil-in-water (w/o/w) techniques call for the use of high shear forces and solvent exposure, both of which are usually detrimental to proteins [14]. Protein encapsulation also creates the possibility for exposure to conditions of high acidity if the protein cannot readily escape the microsphere as the polymer degrades during release. Macrophage colony-stimulating factor [15], fibrolase [16], and interleukin 11 [17] are some examples of acid-sensitive proteins.

There have been published reports on the adsorption and incorporation of rhBMP-2 in porous PLGA microspheres [18,19] and the release profile from a 50:50 copolymer of PLGA, RG503 [20]. The adsorption and incorporation technique provides an alternative to encapsulation. The overall aim of this research was to test the hypothesis that different protein release profiles are achievable from PLGA microspheres by varying the type of PLGA used in the adsorption and incorporation method. Porous PLGA microspheres of similar size and morphology but varying in molecular weight and hydrophobicity were loaded with rhBMP-2 by soaking microspheres in buffered protein solution, filtering the adsorbed microspheres, and drying them. After binding and incorporation were measured, protein release was determined by soaking bound microspheres in a physiological buffer.

MATERIALS AND METHODS

Materials

Lyophilized rhBMP-2 (lots EX2-330 and EX3-002) from Genetics Institute, Inc, Andover, MA, was reconstituted with water for injection and dialyzed first against 10 mM phosphoric acid, pH 2.5, then against 5 mM glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% polysorbate 80, pH 4.5 (referred to as glu buffer), using an 8000 MWCO dialysis membrane (Spectrum Medical Industries, Inc, Houston, TX). Protein solutions were concentrated, as needed, by ultrafiltration on a Diaflo YM 10 membrane (Amicon, Inc, Beverly, MA), stored at 4°C and further diluted with glu buffer as needed. Poly(DL-lactide-co-glycolide) with copolymer ratios of 50/50 or 75/25 (lactide/glycolide %), of various molecular weights, and terminating with either a carboxylic acid or an ester (the ester being formed from the addition of a fatty acid near the end of the polymerization process) were obtained from Boehringer Ingleheim, Ingleheim, Germany (Table 1). In this article, polymers will be referred to by their code names. Polypropylene centrifuge tubes containing inserts with 0.45 μm Durapore filters, used for binding and release experiments, were obtained from Millipore Corp, Bedford, MA (Ultrafree-MC and Ultrafree-CL).

Table 1. Physical Characteristics of Polymers

Polymer	MW*	Mn†	Acid Number‡
RG 501H§	8276	5519	20.8
RG 502	10 754	5014	0.94
RG 502H	10 777	7064	15.1
RG 503	31 281	15 890	0.72
RG 503H	28 022	13 233	4.60
RG 504	55 308	34 128	1.55
RG 504H	53 488	31 273	2.70
RG 752	13 905	10 176	1.15
RG 752H	12 470	7311	14.3

§H identifies the polymer as having carboxylic acid end groups. The 500 series designates a 50:50 PLGA; the 750 series designates a 75:25 PLGA.
*Weight average molecular weight.
†Number average molecular weight.
‡mg KOH/g PLGA.

Preparation of the Microspheres

Porous microspheres were produced using w/o/w double-emulsion technology [18]. Briefly, a water-in-oil dispersion was prepared by introducing a NaCl solution into a methylene chloride solution of the polymer and emulsifying by sonication at room temperature. The dispersion was then introduced into a continuous phase aqueous solution containing polyvinyl alcohol as a surfactant to create a double emulsion. The polymeric microspheres were solidified by solvent removal, achieved by raising the temperature to 40°C. Collected microspheres were washed with water to remove the surfactant and vacuum dried.

Protein Determination

Protein concentrations were determined by high-performance liquid chromatography (HPLC) [18]. Standard curves of rhBMP-2 ranging from 5 to 75 μg/mL yielded linear responses over that concentration range with detection at 214 nm.

Protein Interaction Studies

The rhBMP-2/microsphere adsorption technique has been described [19]. Briefly, the microspheres were suspended in protein solution and allowed to equilibrate before recovery by filtration on a low-protein–binding filter. Subsequent quantification of "free," "bound," and "total" rhBMP-2 associated with the microspheres was carried out by using a simple protein mass balance and the assumption that free protein concentration in the PLGA particles was equivalent to that in the separated rhBMP-2 solution following the filtration step [10]. The total amount of protein added (M_o) to a sample of microspheres was calculated from the product of protein concentration (by HPLC) and solution volume. The microspheres and protein solution were incubated at room temperature (RT) with gentle agitation, then separated by filtration. The weights of the dry microspheres, wet microspheres, and collected supernatant were determined. By mass balance calculation, the amount of protein adsorbed to the microspheres (B_p) is

$$B_p = M_o - F_t - B_c \quad (1)$$

F_t is the total protein in solution after the incubation period and is equal to the product of the protein concentration of the supernatant and the sum of the volume of supernatant (F_s) and the solution sequestered in the pores (F_p). B_t is the amount of protein bound to the tubes used to do the experiment, so the equation above becomes

$$B_p = M_o - (F_s + F_p) - B_c \quad (2)$$

The free protein associated with the microspheres is F_p, the product of the supernatant protein concentration and the volume of solution remaining with the microspheres after filtration. Free protein refers to protein present on the surface and within the pores of the PLGA matrix, whereas bound protein refers to the physically adsorbed rhBMP-2.

The protein/polymer interaction was accomplished by allowing the interaction to proceed for 8, 16, 24, or 48 hours at RT using various protein concentrations (0.17 to 4.4 mg/mL). RhBMP-2 was found to be stable in the glu buffer at RT for at least 1 week. Interaction experiments were done using 20 mg of microspheres and 300 mL of protein solutions. For the release studies, the binding and incorporation was accomplished over 24 hours at RT using 0.2 or 0.4 mg microspheres with 1.5 mL protein solution at a concentration of 0.5 mg/mL.

Protein Release Studies

Release was determined by incubating the loaded microspheres in isotonic phosphate buffered saline (PBS), pH 7.4 with 0.02% sodium azide at 37°C. At 1, 3, 5, 7, 14, 21, 28, and 35 days, the tubes were centrifuged and the remaining wet microspheres weighed. The collected supernatant was assayed for protein concentration and 1.5 mL additional PBS was added to the remaining microspheres for continued incubation.

RESULTS AND DISCUSSION

Polymer and Microsphere Characterization

The physical characteristics of the polymers (and resulting microspheres) used for adsorption, incorporation, and release of rhBMP-2 in this research have been reported [18].

RhBMP-2 Adsorption and Incorporation in Microspheres

Duggirala et al [19] studied the adsorption and incorporation of rhBMP-2 and PLGA RG503 microspheres where the rhBMP-2 concentration ranged from 0.025 to 1.5 mg/mL. The adsorption reached a maximum at 1.0 mg/mL with no increase in binding at 1.5 mg/mL. Adsorption and incorporation of rhBMP-2 into a variety of PLGA microspheres, using protein concentrations from 0.17 up to 0.49 mg/mL, has been published [18]. An attempt was made to increase the levels of bound rhBMP-2 by using a higher protein concentration for the adsorption step (Figure 1). The results indicate binding to RG501H increased with

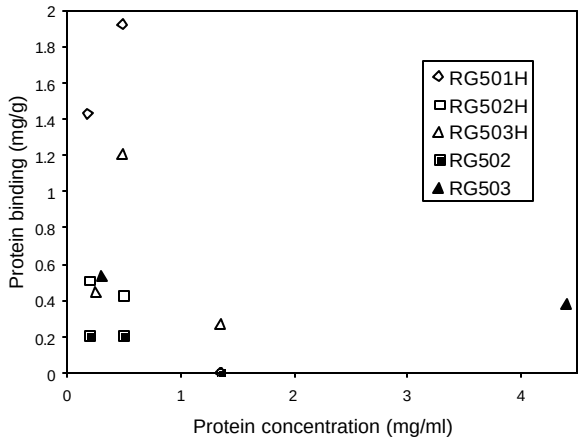

Figure 1. Effect of protein concentration on rhBMP-2 adsorption to PLGA microspheres.

increasing protein concentration (at protein concentrations = 0.49 mg/mL). RG502H showed inhibited or decreased binding in the same protein concentration range. Contributions from protein desorption, if present, were not assessed. RG503H binding behaved similarly to RG501H at protein concentrations = 0.49 mg/mL, but was inhibited or decreased at 1.25 mg/mL. RG502 bound equally at 2 concentrations = 0.49 mg/mL, but showed no binding at 1.25 mg/mL. RG503 bound less well at 4.4 mg/mL than at 0.49 mg/mL. As protein concentrations are increased, aggregation and eventual precipitation occurs. Dimeric or higher order oligomers may have different interactions with the polymers than do single-protein molecules.

To custom load the PLGA microspheres, the binding procedure was varied. Figure 2A shows that increasing the ratio of protein solution to mass of microspheres was advantageous for adsorption to the relatively high binding hydrophilic polymers, RG501H and RG502H. This may be due to a mass effect whereby the higher ratio simply contains more of the rhBMP-2 species capable of binding. Increasing the volume had no effect on the adsorption to the lower binding polymers RG503H and RG502. Figure 2B indicates that introducing the protein solution to RG501H microspheres in 2 steps, allowing some adsorption to take place over an 8-hour period before introducing additional rhBMP-2 for another 16 hours, increases adsorption as compared to a single soaking. This effect is evident, though less so, for RG503H and RG502 microspheres. A possible explanation is that protein multilayers are formed on the microsphere surfaces. RG502H did not show the effect; multiple addition of protein decreased adsorption. Introducing the protein solution in 3 steps, extending to 48 hours in total time of interaction, yielded less adsorbed rhBMP-2 than did introduction of the same rhBMP-2 mass over a single 24-hour period (Figure 2C). Whether protein desorption played a role here was not investigated.

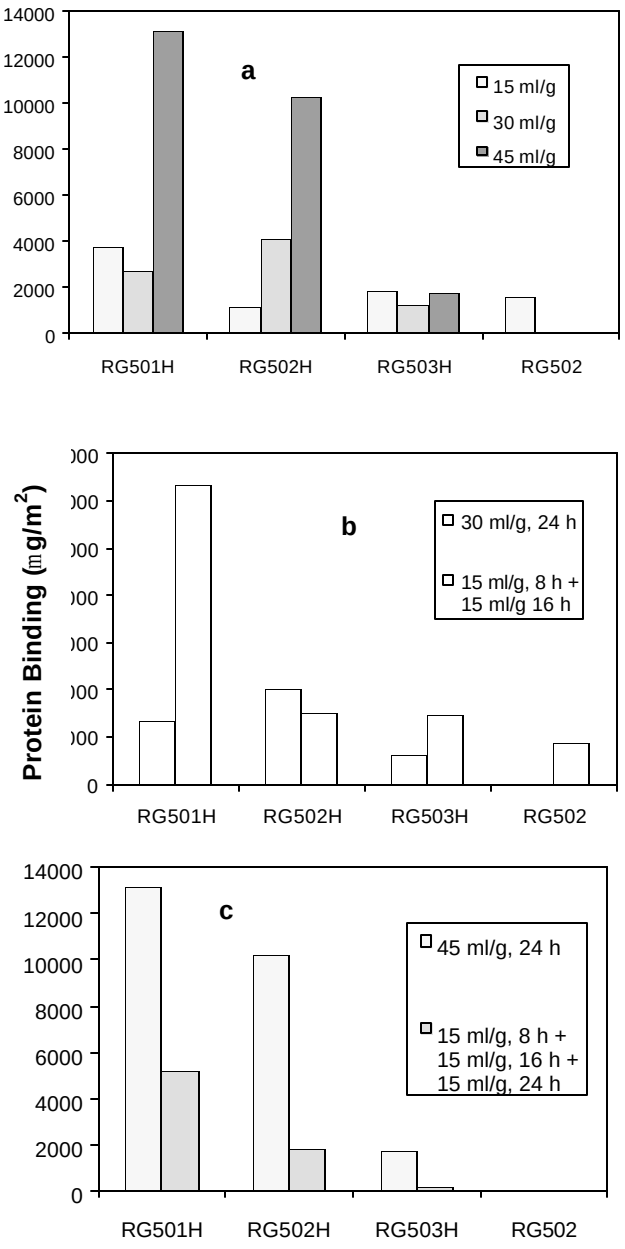

Figure 2. Effects of volume/mass ratio and introduction procedure for binding rhBMP-2 to PLGA microspheres. RhBMP-2 concentration used was 0.4 mg/mL. a, Effect on binding from using 15, 30, or 45 mL rhBMP-2 solution per gram of PLGA. b, Effect of introducing rhBMP-2 at 30 mL/g PLGA in 1 aliquot vs introducing half the solution for 8 hours, followed one half for 16 hours. c, Effect of introducing rhBMP-2 at 45 mL/g PLGA in 1 aliquot vs introducing one third of the solution for 8 hours, followed by one third of the solution for 16 hours, followed by one third of the solution for 24 hours.

Table 2 shows the amount of rhBMP-2 adsorbed, free, and totally incorporated into the PLGA microspheres using the single soaking technique. RG501H bound 9.97 $\mu g/m^2$, RG502H bound 2.40 $\mu g/m^2$, RG503H bound 1.32 $\mu g/m^2$, and RG504H bound 1.06 $\mu g/m^2$. The PLGAs used to prepare those microspheres are essentially identical polyesters, differing only in molecular weight (MW) (from longer repeats of the same structures) and acid number (from differing MW). The trend of increased binding with increased acid number is clear. RG501H, the polymer with the highest acid number, bound so much protein per surface area that at the protein concentrations used for its binding, very little free protein remained. In paired comparisons (RG502H vs RG502, RG503H vs RG503, RG504H vs RG504, or RG752H vs RG752) where the MWs within each pair are similar, the overwhelming majority of structures are chemically identical, and the only difference between them is whether the polymer end groups are a carboxylic function or a long-chain fatty ester, the more hydrophilic polymer bound greater amounts of rhBMP-2. Figure 3 shows the influence of acid number on binding. The data illustrate the trend of hydrophilic polymer binding more rhBMP-2 than hydrophobic polymer and binding less when the composition of the polymer is 75% lactide rather than 50%. The pattern observed in Figure 3 is similar to previously reported results on the influence of acid number and monomer composition on binding [18] where binding was related to ionic interaction between polymer and protein. RhBMP-2 is an alkaline protein (isoelectric point near 9.0). At the pH of the binding experiments, 4.5, the protein will have a net positive charge, whereas the polymers (pK_a values around 4) will be negatively charged. The interaction is strengthened by the increased acidity of the hydrophilic polymers. Duggirala et al [19] has shown previously the contribution of ionic interaction to rhBMP-2 binding to RG503, including diminished binding at increased ionic strength of the binding medium and the ability of high ionic strength buffer to remove bound rhBMP-2 from the PLGA. Evidence of an ionic interaction is shown by the relationship of binding to polymer acid number.

Table 2. Recombinant Human Bone Morphogenetic Protein 2 (RhBMP-2) Incorporation into PLGA Microspheres

Polymer	Bp*	Fp†	Mt‡
RG 501H	9.97	0.09	2.01
RG 502	0.82	0.35	0.56
RG 502H	2.40	0.28	0.71
RG 503	0.43	0.66	0.87
RG 503H	1.32	0.74	1.95
RG 504	0.65	0.30	0.48
RG 504H	1.06	0.76	1.83
RG 752	0	0.26	0.26
RG 752H	1.11	0.32	0.52

*RhBMP-2 bound per specific surface area (mg/m²).
†Free rhBMP-2 incorporated, nonadsorbed into microspheres (mg/g).
‡Total rhBMP-2 associated with the microspheres, bound + free (mg/g).

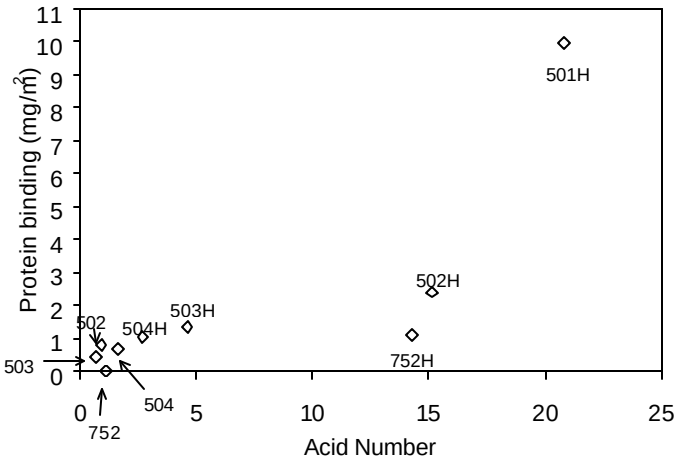

Figure 3. Effect of acid number on rhBMP-2 binding to PLGA microspheres.

RhBMP-2 Release

Figure 4 shows the initial rhBMP-2 released from each type of microsphere. In all cases, regardless of the existence of any adsorbed rhBMP-2, a fraction of protein is released over the first 3 days. The amount of protein released in that period corresponds to the free protein within the microspheres. The quantity of free protein in any of the PLGA microspheres can be adjusted by changing the concentration used for loading or changing the porosity of the microspheres used. However, the early release profile observed is independent of the amount of rhBMP-2 present or the PLGA used.

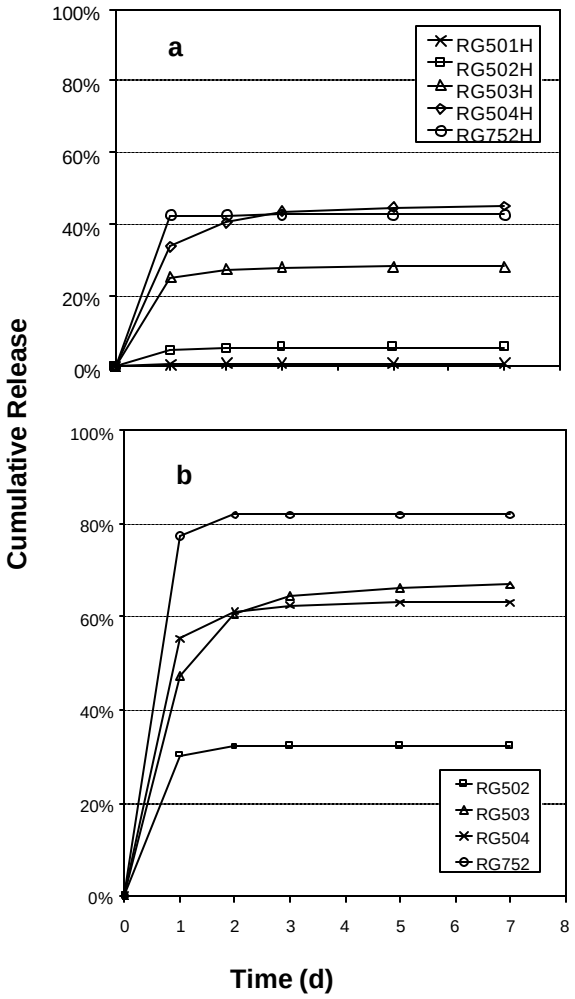

Figure 4. Initial release of rhBMP2. a, From hydrophilic PLGA microspheres. b, From hydrophobic PLGA microspheres.

Figure 5 shows the overall release of rhBMP-2 from 3 types of microspheres, RG501H, RG503, and RG503H. Also shown are the mass changes of the microspheres during the release experiments. (Although RG502H is shown in Figure 5B, RG501H and RG502H have practically identical MW and structure and thus will lose mass in similar profiles.) Following the rhBMP-2 released during the first 3 days, an additional period of release is seen for RG501H and RG503H microspheres and the onset of this second wave of release roughly corresponds to the point where the microspheres have started to physically erode. The bound protein begins to release at that point. Essentially all of the free and bound rhBMP-2 associated with the RG501H microspheres was recovered by 4 weeks. RG503H began releasing bound

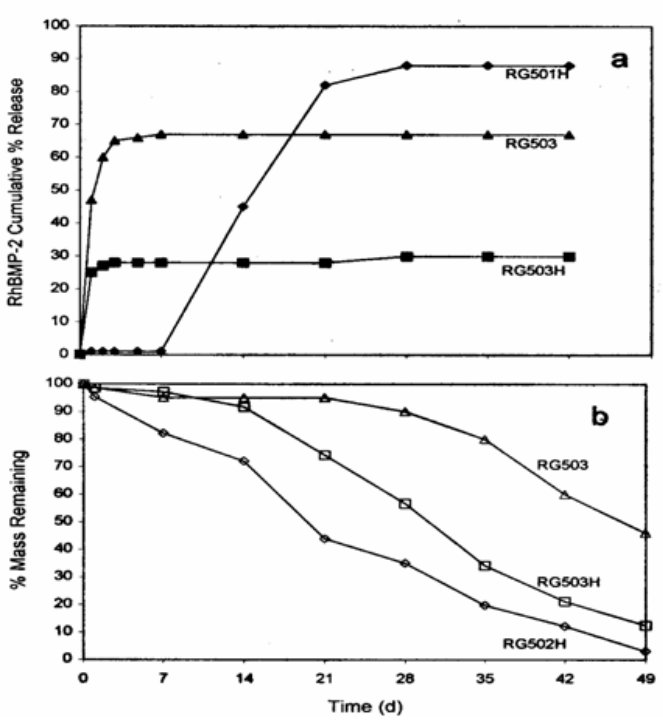

Figure 5. a, RhBMP-2 release and b, polymer mass changes from rhBMP-2 loaded RG501H, RG503H, and RG503 PLGA microspheres.

protein as the polymer eroded, but no protein was detected after 4 weeks because the concentration in the recovery solution had fallen below the detection limit of the assay used. Duggirala et al [20] previously had shown that RG503 microspheres begin to release bound rhBMP-2 at 3 to 4 weeks and continue until at least 8 weeks. Because of the small amounts of rhBMP-2 used in the RG503 release experiment reported here, only the free protein was detected during release. Also, too little material was available at each time point to perform mass balance evaluations. It has been shown previously that bound protein will release in conjunction with polymer mass loss [19,20].

CONCLUSIONS

Porous microspheres of similar size, porosity, and specific surface areas were produced from PLGA polymers that ranged in MW from 8000 to 50 000 g/mol and in acid number from 0.7 to 21. The binding of rhBMP-2 to the microspheres was controlled by the physical chemistry of the polymers used, with the binding capacity correlating with acid number. Increasing the ratio of protein solution to mass of PLGA or adding the protein solution to the microspheres in 2 stages rather than 1 are techniques

to increase the amount of rhBMP-2 adsorbed, particularly for the higher binding, more hydrophilic PLGA polymers. All of the microspheres tested released unbound rhBMP-2 in the first 3 days, and the bound protein released in correlation with degradation and mass loss of the polymers. Some control of rhBMP-2 delivery is thus achievable by selection of the PLGA microsphere carrier.

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