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Physical instability, aggregation and conformational changes of recombinant human bone morphogenetic protein-2 (rhBMP-2)

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ABSTRACT

The influence of two different pH values on the physical stability of recombinant human bone morphogenetic protein-2 (rhBMP-2) in aqueous solution was evaluated in the present work. RhBMP-2 in solution at pH 4.5 or 6.5 was characterized by intrinsic and extrinsic (Nile Red and 1,8-ANS) fluorescence spectroscopy, 90° light-scattering and transmission electron microscopy (TEM). Compared to the pH 4.5 solution, rhBMP-2 at pH 6.5 had (i) a stronger intrinsic fluorescence intensity, (ii) a longer fluorescence lifetime, (iii) a stronger 90° light-scattering intensity, (iv) a stronger Nile Red fluorescence intensity, (v) a higher Nile Red fluorescence anisotropy, (vi) a lower 1,8-ANS fluorescence intensity, (vii) a higher 1,8-ANS fluorescence anisotropy and (viii) a longer 1,8-ANS fluorescence lifetime. Electron microscopy showed that rhBMP-2 at pH 4.5 contained aggregates of about 100 nm in diameter. More and larger protein aggregates (0.1–2 μm) were observed in solution at pH 6.5. Taken together, these results indicate conformational changes and increased aggregation of rhBMP-2 at pH 6.5 compared to pH 4.5, demonstrating a strong influence of pH on rhBMP-2 physical stability. These observations must be considered when developing a delivery system for rhBMP-2.

1. Introduction

Discovered by M.R. Urist in 1965, bone morphogenetic protein-2 (BMP-2) is an important growth factor in bone formation and healing. BMP-2 belongs to the transforming growth factor-β (TGF-β) family, which are multifunctional cytokines that control cell proliferation and differentiation (Celeste et al., 1990). Considering the short half-life of the protein (Hsu et al., 2006; Senta et al., 2009), therapeutic applications of BMP-2 may benefit from a localized delivery system. To be clinically effective, a delivery system should retain BMP-2 at the implantation site for a sufficient time period and sufficient level (Li and Wozney, 2001).

In 2002, the first product containing recombinant human BMP-2 (rhBMP-2) was approved by the Food and Drug Administration (FDA) as an autograft replacement for interbody spinal fusion procedures and by the European Medicines Agency (EMEA) for the treatment of open tibial fractures (Geiger et al., 2003; McKay et al., 2007). To date, FDA and EMEA have approved both indications. The product is marketed as the INFUSE® Bone Graft kit (Medtronic) in the U.S. and as the InductOS® kit (Wyeth) in Europe and consists of lyophilized rhBMP-2 and an absorbable collagen sponge (ACS) carrier for the protein. The collagen sponge retains only a small amount of rhBMP-2 at the repair site (Uludag et al., 1999; Geiger et al., 2003), therefore the applied protein concentration (milligram range) is much higher than the physiologic concentration (nanogram range). To increase the retention of the protein at the implantation site, new types of delivery systems aiming at immobilization of growth factor have been investigated (Luginbuehl et al., 2004).

In order to preserve protein biological activity, particular attention should be given to the design of such a delivery system. Parameters like formulation pH, temperature conditions and shear stress can induce structural changes in proteins during manufacturing, storage, reconstitution and administration processes (Wang, 1999). One of the most common processes that may compromise the stability as well as the desired biological activity of protein drugs is the aggregation of individual molecules, which might lead ultimately to their precipitation (Manning et al., 1989; Middaugh and Volkin, 1992). Aggregates may also trigger other receptors which can result in undesired biological activity, side effects and toxicity (Bucciantini et al., 2002).

The pH of the formulation has a strong influence on the protein aggregation process. A change in pH will change the net charge of

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a protein and thus the balance between attractive and repulsive forces of protein molecules (Chi et al., 2003). At pH values close to the isoelectric point (pI), the relative contribution of attractive interactions between protein molecules increases, favoring precipitation. Regarding rhBMP-2 which pI is 8.2, the literature shows a reduced solubility at pH above 6 (Ruppert et al., 1996; Vallejo and Rinas, 2004). To ensure an adequate solubility, the commercially available rhBMP-2 is formulated in a 5 mM glutamic acid buffer pH 4.5 (Friess et al., 1999a; Schwartz, 2005). However, the combination of rhBMP-2 formulation at pH 4.5 with a delivery system may result in a pH shift toward higher pH. This was observed for instance with collagen sponge (ACS) (Friess et al., 1999a,b) as well as with other delivery systems (Maus et al., 2008; Bergman et al., 2008). An increase in pH might affect the stability of the protein which could influence the bioactivity of the system.

To our knowledge, no information is currently available on pH-dependent physical stability of rhBMP-2. This study investigates the influence of the formulation pH on the aggregation state and conformational changes of rhBMP-2. A commercial formulation of rhBMP-2 at pH 4.5 (InductOs®, Wyeth Pharmaceuticals) was used as a reference and compared with the same formulation neutralized to pH 6.5. This latter pH is close to the pH of the system currently used in clinic after combination of protein formulation with a collagen delivery carrier (Friess et al., 1999a). Various complementary methods, such as intrinsic and extrinsic (hydrophobic dyes Nile Red and 1,8-ANS) fluorescence spectroscopy, light scattering and transmission electron microscopy (TEM) were used to compare rhBMP-2 solutions at pH 4.5 and 6.5. These analytical techniques are well established for the characterization of chemical and physical stability of protein formulations (Pellaud et al., 1999; Lakowicz, 2004; Capelle et al., 2005; Jiskoot and Crommelin, 2005; Demeule et al., 2007b; Hawe et al., 2008).

2. Materials and methods

2.1. Materials

Lyophilized rhBMP-2 from the InductOs® kit (Wyeth Pharmaceuticals, Zug, Switzerland) was stored at −20 °C until use. This lyophilized formulation is composed of rhBMP-2 with 0.5% sucrose, 2.5% glycine, 5 mM L-glutamic acid, 5 mM sodium chloride and 0.01% polysorbate 80 (Friess et al., 1999b; Schwartz, 2005). The solution of rhBMP-2 at pH 4.5 was obtained by reconstitution of the lyophilizate with water. The solution of rhBMP-2 at pH 6.5 was obtained by adding the required quantity of 150 mM sodium hydroxide solution to the pH 4.5 solution. The pH was measured using a Biotrode glass electrode and a pH-meter from Metrohm (Herisau, Switzerland). The concentration of rhBMP-2 at both pH values was 0.75 mg/mL as determined by UV–visible absorbance. Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) and 1,8-ANS (1-anilinonaphtalene-8-sulfonic acid) were purchased from Invitrogen (Luzern, Switzerland). Nile Red was dissolved in ethanol to produce a 100 μM stock solution, which was stored at 4 °C, protected from light. A stock solution of 5.35 mM 1,8-ANS in ethanol was prepared and stored at 4 °C, protected from light. Sucrose, glycine, glutamic acid, sodium chloride, polysorbate 80 and sodium hydroxide were purchased from Fluka (Buchs, Switzerland).

2.2. Steady-state fluorescence spectroscopy

The steady-state fluorescence and steady-state fluorescence anisotropy measurements were recorded with a Fluoromax spectrofluorometer (Spex, Stanmore, UK) at 25 °C in a thermostated cuvette holder. The measurements were performed in a 0.2 cm × 1 cm Hellma quartz cuvette with 400 μL of sample. For all fluorescence measurements, the large side of the cuvette (1 cm) was oriented towards the excitation beam. The intrinsic protein fluorescence, which is essentially due to the tryptophan residues, was monitored between 300 and 450 nm with an excitation wavelength of 280 nm. The spectra were recorded with 0.1 s integration time per 1 nm increment. The excitation and emission slits were set to 0.3 mm and 0.5 mm, respectively. The variation of the fluorescence between two samples of the same protein solution was less than 1%.

Nile Red fluorescence was monitored between 560 and 750 nm, with an excitation wavelength of 550 nm. The spectra were recorded with 0.1 s integration time per 1 nm increment. The excitation and emission slits were set to 1 mm and 2 mm, respectively. Prior to measurement, 4 μL of the Nile Red stock solution was added to 400 μL of the protein solution. 1,8-ANS fluorescence was monitored between 390 and 650 nm, with an excitation wavelength of 372 nm. The spectra were recorded with 0.01 s integration time per 1 nm increment. The excitation and emission slits were set to 0.5 mm and 1 mm, respectively. Prior to measurement 3.9 μL of the 1,8-ANS stock solution was added to 400 μL of the protein solution.

Steady-state fluorescence anisotropy measurements were performed using Glan-Thompson prism polarizers and the anisotropy A was calculated from the equation:

$$A = \frac{I_{0,0} - G \cdot I_{0,90}}{I_{0,0} + 2G \cdot I_{0,90}} \quad (1)$$

where $I_{m,n}$ is the fluorescence intensity at a given wavelength and the subscripts indicate the position of the polarizers in the excitation (m) and emission (n) beams relative to the vertical axis. G is a correction factor: $G = I_{90,0}/I_{90,90}$.

The anisotropy value of the protein fluorescence was calculated from fluorescence spectra between 335 and 360 nm, using an excitation wavelength of 280 nm, with 1 s integration time per 1 nm increment. The excitation and emission slits were both set to 3 mm.

The anisotropy value of the Nile Red fluorescence was calculated from fluorescence spectra between 610 and 640 nm, using an excitation wavelength of 550 nm, with 1 s integration time per 1 nm increment. The excitation and emission slits were set to 2 mm and 4 mm, respectively.

The fluorescence anisotropy value of 1,8-ANS was calculated from fluorescence spectra between 465 and 500 nm, using an excitation wavelength of 372 nm, with a 1 s integration time per 1 nm increment. The excitation and emission slits were set to 1 mm and 2 mm, respectively.

2.3. Steady-state fluorescence lifetime

Fluorescence lifetimes were measured using time-correlated single-photon counting (TCSPC) on an IBH 5000U fluorescence lifetime spectrophotometer (Jobin Yvon Horiba, New Jersey, USA). NanoLED sources with excitation wavelengths at 279 nm, 371 nm and 560 nm were used to measure the fluorescence lifetimes of protein, Nile Red and 1,8-ANS, respectively. The emitted photons were counted at 345 nm, 625 nm and 480 nm for protein, Nile Red and 1,8-ANS, respectively. For all lifetime measurements, the small side of the cuvette (0.2 cm) was oriented towards the excitation beam. Data analysis was performed using the DAS6 program (Jobin Yvon Horiba, New Jersey, USA). The measured fluorescence intensity decay was deconvoluted with the instrument response function, as measured using a dilute suspension of colloidal silica (Ludox, Aldrich, Milwaukee, USA). The calculated fluorescence intensity decay with time was fitted with a multi-exponential model. The intensity-weighted average fluorescence lifetime, τ_F, was calculated from the individual fluorescence decay times τ and the normalized pre-exponential values α using the equation