Imaging Therapeutic Proteins in Gelatin for Controlled Drug Release

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Introduction

In response to an event disrupting bone function, proteins are secreted to initiate healing. Disease and other factors can diminish the ability of bone to self-repair. Therefore, augmentation through the use of artificial bone may be required. The addition of therapeutic proteins, or growth factors, to engineered bone constructs is believed to elicit a more natural response at the defect site and to decrease healing time [1]. Drug delivery vehicles must be carefully engineered in order to optimize the dose and dose rate and these parameters must be monitored over time and space. One technique used to measure the pharmacokinetics of growth factors is radioactive labeling [2]. However, this only yields an average value over the sample; it does not provide a 3D description. The radioactivity of the label adds further complications.

Preliminary results from the 2BM beam line at the Advanced Photon Source demonstrate the feasibility of imaging proteins using x-ray microcomputed tomography (micro-ct) for drug release applications. Soybean trypsin inhibitor (STI), a model protein for the growth factor BMP-2, was labeled with either gold nanoparticles or nonradioactive iodine to provide x-ray absorption contrast. Gelatin microspheres, engineered to produce a controlled release of the growth factor, were loaded with one of the labeled proteins and incorporated into a gelatin sheet. Both the loaded microspheres and the sheet containing the microspheres[*] were imaged. Quantities as low as 1 µg protein/mg gelatin were detected. This technique may offer an alternative method to generate a 3D representation of the pharmacokinetics, rapidly, and without radioactive tracers. Furthermore, the technique has potential for *in vivo* applications [3,4]. Results will have significant impact on clinical design of bone implants.

Methods and Materials

Preparation of Carrier Matrix – Gelatin Microspheres

Gelatin microspheres (pI = 9.0) were created by a water-in-oil emulsion technique [5]. Briefly, 10 mL of a 10 wt% gelatin solution was added to olive oil to create a water-in-oil suspension. The solution was mixed at 40 ºC and then cooled to 4ºC to precipitate the microspheres. The microspheres were washed several times with acetone and collected by centrifuge. After allowing the acetone to evaporate overnight, the microspheres were crosslinked using 500 µL gluteraldehyde per 20 mg microspheres, washed with 100 mM glycine, and freeze-dried. Microspheres made by this method are irregular in shape and can range in size from 20-100 µm.

Protein Labeling

The model protein, STI, was used in lieu of the growth factor of interest, BMP-2, because of the similar molecular weight and number of amine groups, and the significantly lower cost, as are shown in Table I. The number of aromatic groups did not match as well. The amine groups provide potential sites for gold conjugation by 2-Iminothiolane•HCl, (Traut's reagent), which reacts with primary amine groups ($-NH_2$) to introduce sulfhydryl groups (-SH), which bind strongly to the surface of the gold [6,7]. The aromatic groups provide sites for iodination of the protein [8]. In particular, chloramine-T has strong oxidizing properties that readily lead to the formation of the required electrophilic halogen species that result in iodine incorporation into target molecules [8]. The data reported here includes: (1) microspheres with unlabeled STI as the control; (2) microspheres with Au-labeled STI (µ-Au-STI); and (3) microspheres with I-labeled STI (µ -I-STI).

Table I. Comparison of BMP-2 to model protein, Soybean Trypsin Inhibitor (STI)

Protein	MW (kD)	pI	(*-NH2)/ protein*	Aromatic group/protein	Price/µg ($)
BMP-2	25	8.5	19	21	20
STI	21.5	4.6	20	6	.001

Gold Conjugation

A weight of 2.5 mg of the unlabeled STI was dissolved in a non-amine buffer with pH 8.0. Next, a 5-fold molar excess of Traut's reagent (40 µL) was added to the protein and incubated for 1 hour at room temperature. The reagent excess was separated from the thiolated protein and the protein concentration was determined using the BCA Protein Assay measuring the absorbance at 562nm with a spectrophotometer.

The pH of the gold colloid was adjusted to the isoelectric point of the protein to maximize the binding and the stability of the complex [9]. Following the ratio suggested by the manufacturer, 35 mL colloidal gold was added to 1 mL of the thiolated protein and the solution was incubated overnight at 4°C. After the gold-protein complex was centrifuged and the supernatant discarded, the gold-labeled protein was diluted to 1 mL PBS, pH 7.4. Finally, the concentration was measured as described above. The gold conjugation procedure is described in more detail in [10].

Iodination

First, 1 mg of the protein was dissolved in 1 mL of PBS. The protein/PBS solution was then mixed with a 5M NaI solution. The reaction initiated and stopped using 0.9 mM Chloramine-T in PBS and 21 mM sodium metabisulfite, respectively. The iodinated protein solution was poured through an ion exchange column in a 2.5 mL syringe equilibrated with PBS, and then incubated overnight at 4°C [11,12]. The labeled protein concentration was measured as described above and the iodination procedures are described in more detail [10].

Protein Incorporation

A volume of 120 µL of each labeled proteins was added to 12 mg of freeze-dried gelatin microspheres. After two hours at room temperature, the microspheres in solution were diluted in 210 µL of PBS. Samples were taken from the two microsphere solutions and used as the µ-Au-STI and µ-I-STI samples. The

[*] This report focuses on results from the microspheres. Details on the imaging of the sheet containing the loaded microspheres can be found in [10].

final concentration of the labeled protein in the microspheres was approximately 0.75 µg protein/mg gelatin microspheres.

Microcomputed Tomography

Micro-CT experiments were conducted at beamline 2-BM, of the Advanced Photon Source, Argonne National Laboratory using synchrotron radiation. Full details of the experimental set-up can be found in Wang *et al.* [13]. Prior to imaging, all samples were deposited on the sample mount and dried using acetone. Imaging wet specimens was not possible due to sample movement induced by beam heating. The incident monochromatic energy was 19.1 keV and the data acquisition time for a 1024x1024 single projection with 720 total projections was approximately 10 minutes. The field of view was 2.5 mm and, under these conditions, the spatial resolution was 2.4 µm/voxel. The microspheres were piled into a column of approximately 1 mm in diameter for imaging. The data collected were reconstructed as a series of cross-sectional images with the measured linear attenuation associated with each voxel element.

Data Sampling

The number of voxels for each attenuation value was plotted in the form of a histogram for the entire sample. Specific regions were sampled locally and histograms made in order to distinguish the minute quantities of protein and therefore, regions containing the gold or iodine from the rest of the specimen. For data rendering, we used Amira™ from Indeed - Visual Concepts GmbH. Locally sampled regions for the microspheres included control unlabeled STI incorporated into gelatin microspheres and regions believed to contain the Au- or I-labeled protein. For the analysis of the microspheres, 15 representative volumes containing 3 x 10^2 voxels each were sampled from different cross sections of the same specimen. In order to obtain a representative region of the microspheres containing the labeled protein without a large volume of background, small volumes were sampled.

Confocal Microscopy

Confocal microscopy was used to image the Au-labeled microspheres for comparison to micro-ct results. Samples were mounted in glycerol/PBS (9:1, refractive index = 1.45) and imaged using a Leica SP2 Confocal and Multiphoton microscope equipped with a Krypton Argon Laser. Data was collected from two channels simultaneously; one channel recorded the autofluorescence from the gelatin, the other the reflected light from the gold. Image volumes were assembled from a vertical series of images with focus control provided by a precision microstepping motor. The iodinated protein will not fluoresce or reflect light and therefore could not be imaged in this manner.

Results

A representative grey scale image of the µ-Au-STI, the focus of this report, is shown in Fig. 1. Results were similar for the gold and iodine data. Localized bright spots or high contrast regions near the outside of the microspheres represent regions of higher attenuation and are thus indicative of the presence of Au (or I). A representative region is enlarged in the inset (see black arrow and inset). In a paper by Wagoner Johnson *et al*, it was shown that the bright spots visible in the image (and similarly for the sheets containing protein-loaded microspheres [10]) were in fact associated with the labeled protein and not with artifacts typical to micro-ct. Examples of these include concentric rings and line artifacts [10]. The image shown here does not contain especially prominent artifacts.

The attenuation data from sample volumes of the µ-Au-STI, µ-I-STI, and µ-STI samples are shown in Figure 2. A 4th order Butterworth filter was applied to reduce the high frequency noise. The data from the µ-STI have a relatively symmetric peak, centered around 25 cm^{-1}. Both the µ-Au-STI and the µ-I-STI have relatively asymmetric peaks, with more voxels with higher attenuation values for the same sampled volume. This data also strongly suggests that microgram quantities of Au- or I-labeled proteins can be detected in the gelatin microspheres using micro-ct.

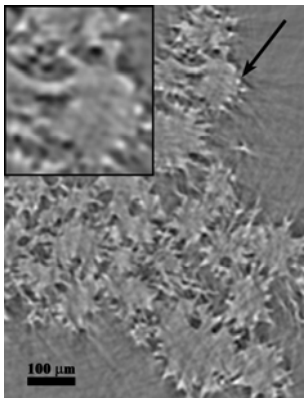

Fig. 1. Cross-sectional micro-ct image of microspheres containing Au-labeled STI. The inset shows an enlargement of the region indicated by the black arrow. Note the concentrrated bright spots, indicative of a higher attenuation, and therefore the presence of the gold nanoparticles. Iodinated samples showed similar results.

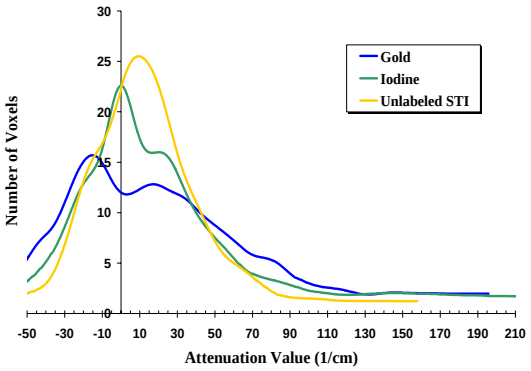

Fig 2. Micro-ct data for gelatin microspheres containing Au- or I-labeled protein in the form of a histogram. These data were obtained by sampling small volumes of select regions in cross-sectional images.

Confocal microscopy was used to support micro-ct results for the µ-Au-STI samples and representative images are shown in Figures 3. Gelatin microspheres without STI and microspheres with unlabeled STI were used as controls. The top row of images in Figure 4 shows composite 3D images from both the autofluorescent microsphere and the reflected light from the gold nanoparticles. Autofluorescence is observed in the microspheres due to the gluteraldehyde crosslinking. The bottom row of images shows only the reflected light. Clearly, the sample containing the gold reflects more light than the control samples and the distribution of the additional reflected light is consistent with the results obtained from micro-ct; the

gold is concentrated near the outer surface of the microsphere and in the pockets or folds of the irregular surface of the sphere. Some reflected light is also present in the control samples, and this is caused by the index of refraction mismatch between the gelatin/protein combination and the mounting medium. A mounting medium with a better match is being investigated.

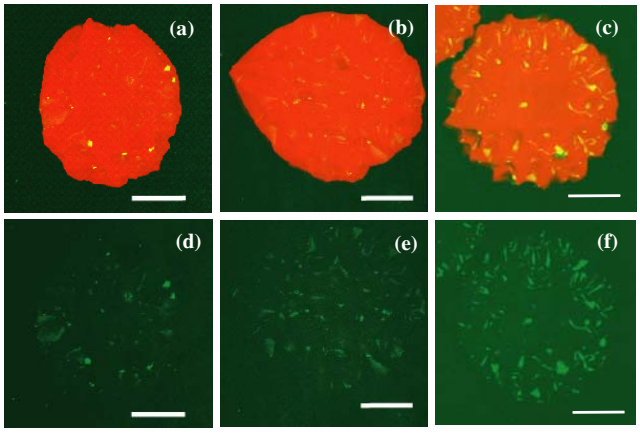

Fig. 3. Representative confocal images of the gelatin microspheres. Images (a-c) are composite images made from the fluorescent and reflected light. Images (d-f) are the show the reflected light only. (a) An empty microsphere, (b) a microsphere containing unlabeled protein, and (c) a microsphere containing the Au-labeled model protein, soybean trypsin inhibitor. Scale bar is 40 microns.

Discussion and Conclusions

The study demonstrates the use of micro-ct for detecting Au- and I-labeled therapeutic proteins for controlled drug release applications. Micro-ct has advantages over alternative techniques because it is non-destructive, little sample preparation is required, and millimeter-sized samples can be imaged with resolution less than 3 μm. The advantage of using gold nanoparticles as a drug label is that the particles are nontoxic, nonradioactive, and are less disruptive to the activity of the protein [9,14] than is the iodine (radioactive or nonradioactive).

The results from this study specifically show that microgram quantities of Au- and I-labeled soybean trypsin inhibitor, a model protein for the bone inducing growth factor BMP-2, per milligram of gelatin can be detected using synchrotron micro-ct. There is no apparent difference in measured attenuation for the gold versus the iodine label, which we rationalize in the following way. In the case of nano-sized particles, a single particle attaches to each protein molecule [15], while a total of six iodine atoms can theoretically attach [16,17]. Even though the relative linear attenuation of gold relative to iodine is high (10:1), we speculate that multiple sites per protein molecule were occupied by iodine, while the total protein concentration in Au- and I-labeled samples was equal. As a result, samples containing iodine showed similar attenuation values as those containing gold. The advantage of using the gold is that it is less likely to denature the protein and can be detected using other methods, such as with confocal or transmission electron microscopy, in order to confirm results. Here, confocal microscopy showed results consistent with those from the micro-ct experiments.

Micro-ct is a non-invasive technique and there is significant potential for using this method of drug detection *in vivo*. The latter is especially true when considering drugs used in higher concentrations, for example in milligram rather than microgram quantities [3,4], and as the resolution of computed tomography improves with technological advances.

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References

[1] M. Joyce, S. Jingushi, M. Bolander Ortho Clin N Amer 21 199-209 (1990).
[2] Y. Yu, J.-L. Yang, P. J. Champman-Sheath, W. R. Walsh J Biomed Mater Res 60 392-397 (2002).
[3] A. Szymanski-Exner, N. T. Stowe, K. Salem, R. Lazebnik, J. R. Haaga, D. L. Wilson, J. Gao, J Pharm Sci 92 289-296 (2003).
[4] A. Szymanski-Exner, N. T. Stowe, R. S. Lazebnik, K. Salem, D. L. Wilson, J. R. Haaga, J. Gao, J Control Release 83 415–425 (2002).
[5] Y. Tabata, Y. Ikada, K. Morimoto, H. Katsumata, T. Yabuta, K. Iwanaga and M. Kakemi, J Bioact Compat Pol 14 371-384 (1999).
[6] A. Kudelski, J Raman Spectrosc 34 853-862 (2003).
[7] K. Prime, G. Whitesides, Science 252 1164-1167 (1991).
[8] D. S. Wilbur, Bioconjugate Chem 3 (1992).
[9] M. A. Hayat Colloidal Gold Principles, Methods and applications, in, 1989, pp. 38.
[10] A. Wagoner Johnson, N. Juan Serrano, A. Whittington Morgan, R. D. Jamison, Y. B. Choy, H. Choi, K. Kim, F. DeCarlo, Macromolecular Symposia (in press, 2005).
[11] D. S. Wilbur, S. W. Hadley, M. D. Hylarides, P. G. Abrams, P. A. Beaumier, A. C. Morgan, J. M. Reno, A. R. Fritzberg, J Nucl Med 30 216-226 (1989).
[12] A. E. Bolton, W. M. Hunter, Biochem J. 133 529-539 (1973).
[13] Y. Wang, F. DeCarlo, D. Mancini, I. McNulty, B. Tieman, J. Bresnahan, I. Foster, J. Insley, P. Lane, G. v. Laszewski, C. Kesselman, M.-H. Su, M. Thiebaux, Rev Sci Intstrum 72 2062-2068 (2001).
[14] S. L. Goodman, G. M. Hodges and D. C. Livingston, Scan Electron Microsc 11 133 (1980).
[15] J. E. Beesley Colloidal gold : a new perspective for cytochemical marking, in: J. E. Beesley (Ed.), Microscopy Handbooks, Oxford University Press, New York, 1989.
[16] National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, Bethesda, MD.
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