A New Microphotolysis Based Approach for Mapping the Mobility of Drugs in Microscopic Drug Delivery Devices

Stefaan C. De Smedt,[1,3] Tom K. L. Meyvis,[1] Patrick Van Oostveldt,[2] and Joseph Demeester[1]

Received March 11, 1999; accepted July 8, 1999

KEY WORDS: microscopic dosage forms; drug delivery; microphotolysis; photobleaching; confocal scanning microscopy; diffusion coefficients.

INTRODUCTION

In the last decade, interest in drug targeting and controlled drug delivery has oriented pharmaceutical research toward developing micro- and nano-scopic devices which host the drug (1,2). Publications on micropellets, microcapsules, microgels, polymeric microspheres, microemulsions, liposomes, polymeric nanoparticles and polymeric micelles are numerous. The characterization and optimization of drug release from such microscopic dosage forms is a major topic in physico-pharmaceutical research (2). In addition to studying drug release, physico-chemical characteristics such as particle size distribution, morphology and surface charge are often considered.

To study the processes which occur inside microscopic dosage forms and the influence they have on drug release, new methods are required. NMR imaging (3), photographic methods (4) and video imaging (5) were succesfully applied in studying the internal solvent distribution during swelling of macroscopic dosage forms, such as tablets. However, limited resolution prevents applying those techniques on microscopic dosage forms. Scanning electron microscopy (SEM) and Fourier transform infrared microscopy were used to study internal drug distribution in pellets (6) and hydrogels (7). However, SEM requires samples to be frozen which might introduce artifacts in the internal structure. Interesting work on internal phenomena occuring in microspheres and pellets was performed using confocal scanning laser microscopy (CSLM). Pereswetoff-Morath *et al.* (8) showed by CSLM that the internal distribution of insulin in sephadex microspheres was governed by the cut-off limit of the spheres while Cutts *et al.* (9) developed a CSLM based method for quantifying the minocycline release from spherical pellets (250 μm < size < 400 μm). They studied, non-invasively, the changing internal minocycline distribution during release from the pellets which allowed them to predict the observed biphasic release profile.

The aim of this study was to evaluate whether a recently developed CSLM based method, called scanning microphotolysis (SCAMP) (10), would allow screening the mobility of fluorescent labeled drugs, being one of the major driving forces for release, at arbitrary locations inside small pharmaceutical microspheres.

MATERIALS AND METHODS

Materials

Dextran-methacrylate (dex-ma), used to prepare dex-ma microspheres, was synthesized and characterized as described in detail elsewhere (11). Dex-ma microspheres were prepared by radical polymerization (using N,N,N′,N′-tetramethylene-ethylenediamine and potassium persulfate) from a dex-ma/polyethyleneglycol (PEG) emulsion (12). The concentration of the dex-ma solution (in phosphate buffer at pH 7) was 10% (w/w). The degree of substitution of dex-ma, being the number of methacrylate molecules per 100 glycopyranosyl units, was 4. The concentration of the PEG solution (in phosphate buffer at pH 7) was 24% (w/w) while the average molecular weight of PEG was 10 000 g/mol (Merck). One batch of microspheres ("FD148-dex-ma microspheres") was prepared in the presence of fluorescein isothiocyanate labeled dextran 148 000 g/mol (FD148; 2 mg/mL in phosphate buffer at pH 7) which was used as a model for macromolecular drugs. A second batch of microspheres was loaded with FITC by submersion of the dex-ma microspheres (after complete preparation) in a FITC solution (0.01 mg/mL in phosphate buffer at pH 7.2). Both FD148 and FITC were from Sigma. SCAMP experiments on suspensions of dex-ma micropsheres (in phosphate buffer at pH 7) were performed on the day they were prepared.

Methods

In microphotolysis (MP) based techniques (13), often called fluorescence recovery after photobleaching (FRAP), the mobility of a fluorescent molecule is measured by bleaching (i.e. photolyzing) the fluorescent molecules (using an intense laser beam) moving in the focal area of the laser beam (Fig. 1. A → B). Immediately after the short bleaching process, typically about ten milliseconds, a highly attenuated laser beam measures the recovery of the fluorescence in the photobleached area due to diffusion of fluorescent molecules from the surrounding unbleached areas into the bleached area (Fig. 1. B → C). The characteristic diffusion time (T_D), a measure for the diffusion coefficient, and the fractions of respectively immobile and mobile fluorescent molecules can be derived from the fluorescence recovery in the bleached area (Fig. 1. D, (13)). The mobile fraction, R, is defined as:

[1] Faculty of Pharmacy, University of Ghent, 9000 Ghent, Belgium.
[2] Faculty of Agricultural and Applied Biological Sciences, University of Ghent, 9000 Ghent, Belgium.
[3] To whom correspondence should be addressed (e-mail: stefaan.desmedt@rug.ac.be)

Abbreviations: AOM, acousto optic modulator; CSLM, confocal scanning laser microscope; dex-ma, dextran mathacrylate; FRAP, fluorescence recovery after photobleaching; F, normalized fluorescence intensity; $F(i)$, normalized fluorescence intensity of the bleach spot before bleaching; $F(0)$, normalized fluorescence intensity of the bleach spot just after bleaching; $F(\infty)$, normalized fluorescence intensity of the bleach spot at infinite time after bleaching; SCAMP, scanning microphotolysis; MP, microphotolysis; R, mobile fraction; SEM, scanning electron microscopy; T_D, characteristic diffusion time.

0724-8741/99/1000-1639$16.00/0

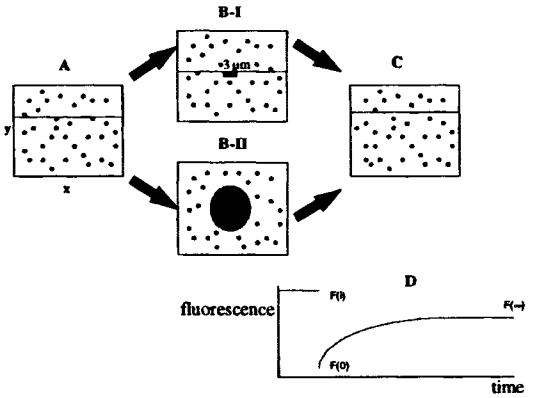

Fig. 1. Principles of conventional MP and SCAMP. The dots represent the fluorescent molecules in the x-y focal plane of a sample. A) represents the focal plane before bleaching. B) represents the focal plane immediately after bleaching a 3 μm segment (by SCAMP; B-I) or a larger spot (by conventional MP; B-II). C) represents the focal plane at the end of the recovery process. D) shows how the fluorescence recovery curve looks when a part of the fluorescent molecules are immobile. The dotted line in A, B-I and C is the x-line of the focal plane where scanning occurs in the SCAMP experiment.

$$R = \frac{F(\infty) - F(0)}{F(i) - F(0)} \quad (1)$$

where $F(i)$ is the normalized fluorescence intensity of the bleach spot before bleaching, $F(0)$ is the normalized fluorescence intensity of the bleach spot just after bleaching and $F(\infty)$ is the normalized fluorescence intensity of the bleach spot at infinite time after bleaching. The normalized fluorescence recovery is obtained by dividing the fluorescence intensity (in the bleached area) at each time point by the corresponding fluorescence intensity in a non-bleached region of the sample. This allows correction of the fluorescence recovery curve for the low amount of bleaching which occurs during the recovery process. This correction is necessary as the FRAP theory assumes that the fluorescence recovery profile is only due to diffusion and is not affected by additional photobleaching during the recovery process.

In this study, a MP variant (SCAMP) was evaluated. The "results and discussion section" explains how SCAMP proceeds. We installed SCAMP on a Bio-Rad MRC1024 CSLM following the work of Wedekind et al. (10,14) who originally developed SCAMP on a Leica CSLM. A 40× oil immersion objective and a powerful 2 W argon laser (Spectra Physics 2017), necessary for obtaining sufficient photobleaching during the extremely short photobleaching times, were used in the SCAMP experiments on dex-ma microspheres. The wavelength of the laser beam, also during bleaching, was 488 nm.

RESULTS AND DISCUSSION

The availability of laser light scanning microscopes opened new perspectives for MP methods. In MP experiments using a conventional (non scanning) light microscope, a stationary (laser) beam was focused on the sample during both the bleaching process as well as the recovery period. The stationary position of the laser beam results in a photobleached area which has a circular geometry. Although non scanning light microscopes technically yield an irradiated area of ≈2 μm in diameter, broadening of the bleach spot often occurs due to the stationary laser beam (schematically illustrated in Fig. 1. B-II). As injectable microspheres often have a typical size of a few μm, the bleach spot of conventional MP setups tends to be too large as the fluorescent molecules in a big part of the microsphere become photobleached. This partially limits the use of conventional MP in studying the mobility of fluorescent labeled drugs inside such microscopic delivery devices. In SCAMP, a new MP approach using a scanning laser microscope, bleaching occurs during scanning the sample by switching between low monitoring and high photobleaching laser intensity levels in less than a microsecond using an acousto-optical modulator (AOM). The combination of bleaching during scanning, and the use of the AOM which generates extremely short photobleaching pulses, prevents the broadening of the bleach spot which occurs in conventional MP due to longer photobleaching times and the stationary laser beam. SCAMP allows bleaching spots of less than a micrometer in the sample. Moreover, using a confocal microscope allows fluorescence detection not only at the surface but also at an arbitrary depth in the sample, without any interference by scattered radiation from out-of-focus levels of the specimen (as encountered in a conventional microscope). Both the confocal and scanning features of the microscope allow photolyzing microregions at well defined locations within a sample.

In this study, SCAMP experiments were performed at approximately 10 μm below the surface of the dex-ma microspheres. It occured experimentally as follows. First, the fluorescence along one x-line of a middle plane of a dex-ma microsphere was measured by scanning this line 400 milliseconds (Fig. 1. A-dotted line). Second, a 3 μm segment on this x-line was selected to be bleached (Fig. 1. B-I). The length, position, as well as the number of segments are freely selectable by the SCAMP software. The photobleaching of this segment occured at the time the laser beam scanned over this segment accompanied by a temporarily strong increase in the intensity of the laser beam. The ratio between monitoring and photobleaching intensity levels of the laser beam was 1:500. To measure the fluorescence recovery in the bleached stripe, a strongly attenuated laser beam scanned along the selected x-line for approximately 4 seconds.

Fig. 2 shows the confocal images of a middle plane in a "FD148-dex-ma microsphere" respectively before and 2 minutes after bleaching the 3 μm segment. The latter image shows the bleach spot remains black indicating that, after 2 minutes, no fluorescence recovery occured in the bleached region of the microsphere. Fig. 3 (curve A) shows the fluorescence in the bleached segment of this experiment did not recover which allowed us to conclude that, within the time scale of the experiment, the "large" FD148 chains were completely immobilized in the region of the dex-ma microsphere under investigation. The low photochemical stability which favors sufficient bleaching in MP experiments, the relatively high absorbtion and the high quantum efficiency of fluorescein and its derivatives are the main reasons why FITC has been chosen as the fluorescent label in this study.

While FD148 chains could be sterically entrapped in the dex-ma polymer network as they were present during the formation of the microspheres, this could not occur for small FITC molecules when loaded into dex-ma microspheres by submersion of the fully polymerized dex-ma spheres into a FITC

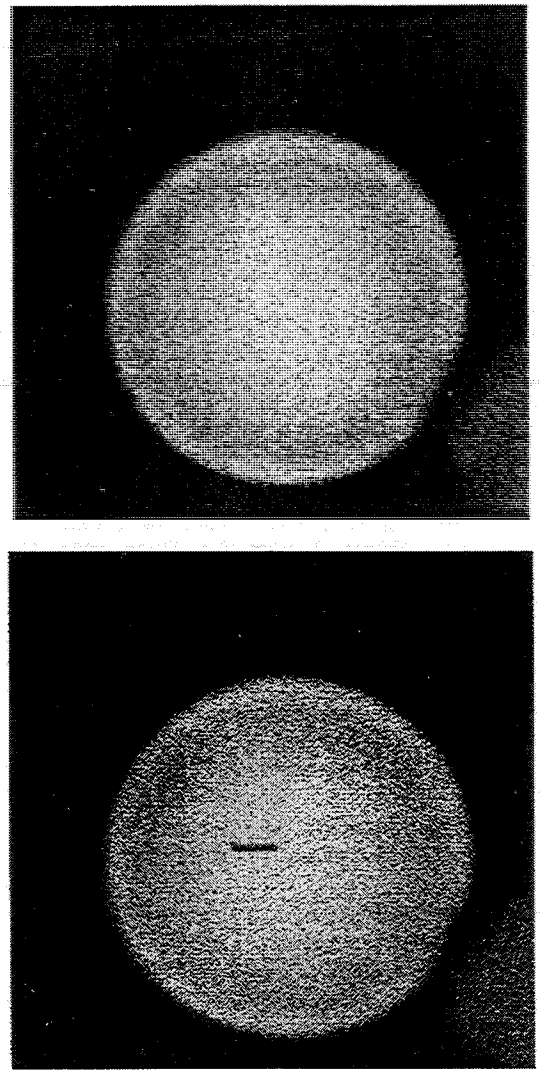

Fig. 2. Confocal images of a middle plane of a "FD148-dex-ma microsphere" before (upper) and after bleaching (under) a stripe of 3 μm at approximately 10 μm under the surface of the microsphere. The diameter of the dex-ma sphere is approximately 25 μm.

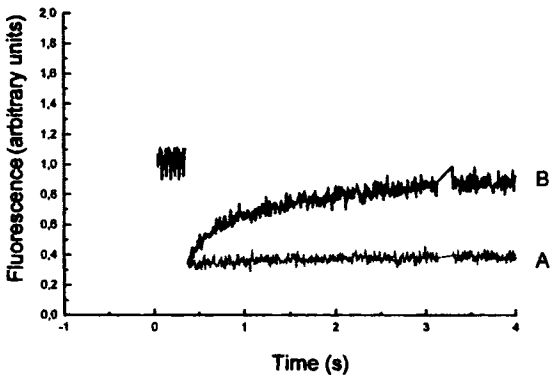

Fig. 3. Fluorescence recovery curves of FD148 in "FD148-dex-ma microspheres" (A) and FITC in dex-ma microspheres loaded with FITC by submersion in a FITC solution (B).

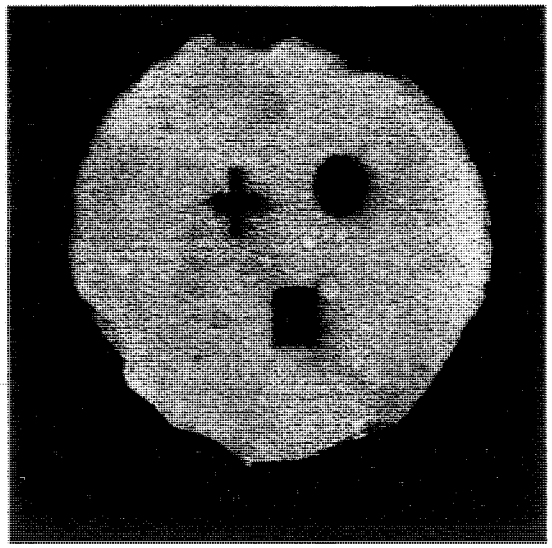

Fig. 4. Confocal images of a middle plane of a "FD148-dex-ma microsphere" after bleaching an arbitrary geometry by SCAMP.

solution. In this case, a complete fluorescence recovery was expected and experimentally confirmed. Fig. 3 (curve B) shows that FITC molecules located around 10 μm under the surface of the microsphere remain mobile.

Besides the technical ability of photobleaching small segments in a sample, compared with conventional microscopes, using scanning microscopes is straightforward to specifically select that microregion in the sample where bleaching has to occur as the laser beam can be locally positioned. Moreover, as the length, position as well as the number of segments are freely selectable in SCAMP experiments, any kind of geometry in the sample can be photobleached. Figure 4 shows the confocal image in a middle plane of a "FD148-dex-ma microsphere" 2 minutes after bleaching a cross, a circle and a rectangle in the microspheres. The possibility of bleaching an arbitrary geometry and studying the recovery inside such regions might be especially useful in studying mobility and interactions in microscopic heterogeneous regions which might be observed inside microscopic dosage forms as in Fig. 4.

On one hand, SCAMP provides us with direct information on the mobility of the drug which is important in the understanding and optimization of the release process. On the other hand, as the mobility of a drug in a microsphere might be related to the local morphology, measuring its dynamic properties at different places, may indirectly provide information on the internal morphological structure. This is important in polymer blends where phase separation may result in large heterogeneities in the internal structure of the devices. Also, as polymer degradation in microspheres may alter the dynamics of entrapped (macro)molecules, mapping the mobility by SCAMP could be an interesting alternative to observe whether the microspheres degrade homogeneously or heterogeneously.

CONCLUSIONS

To our knowledge, this is the first time that the feasibility of a MP based method studying the dynamic behavior of fluorescent labeled molecules inside injectable pharmaceutical microspheres has been evaluated. We have shown that, due to the high

spatial bleaching resolution, SCAMP may become an attractive nondestructive alternative to screen the mobility of fluorescent drugs at freely selectable places in microscopic dosage forms like polymer microspheres. Especially, by the use of a confocal miocroscope, the mobility can be mapped not only in the outer surface regions but also in the inner regions of microscopic dosage forms. In recent years CSLM has become a rather routinely used technique in the field of drug delivery and biopharmacy to study drug distribution in delivery systems and cells. We feel that SCAMP, which can be rather easily installed on any CSLM, makes full use of the advantages of CSLM. As established methods exist to attach fluorescent markers on macromolecular therapeutics like peptides, proteins and genes, SCAMP may offer new technical perspectives especially for macromolecular drug delivery research, which may lead to new insights into the dynamics governing macromolecular drug delivery.

ACKNOWLEDGMENTS

Stefaan De Smedt is a postdoctoral fellow of FWO-Vlaanderen. Tom Meyvis is a doctoral fellow of IWT. The confocal microscope and fluorimetry infrastructure was financially supported by FWO-Vlaanderen and BOF of the University of Ghent (UG). The installation of SCAMP was financially supported by FWO-Vlaanderen. Dex-ma was synthesized by the group of Professor W. Hennink while Dr. P. Wedekind and Dr. U. Kubitscheck supported us with the implementation of SCAMP. All of them, as well as FWO-Vlaanderen, IWT and UG are gratefully acknowledged.

REFERENCES

1. L. M. Sanders and R. W. Hendren. *Protein Delivery: Physical Systems*, Plenum Press, New York, 1997.
2. R. H. Müller. *Colloid Carriers for Controlled Drug Delivery and Targeting*, CRC Press, Boca Raton, 1991.
3. A. R. Rajabi-Siahboomi, R. W. Bowtell, P. Mansfield, M. C. Davies, and C. D. Melia. Structure and behaviour in hydrophilic matrix sustained release dosage forms: 2. NMR-imaging studies of dimensional changes in the gel layer and core of HPMC tablets undergoing hydratation, *J. Contr. Rel.* **31**:121–128 (1994).
4. P. Colombo, R. Bettini, G. Massimo, P. L. Catellani, P. Santi, and N. A. Peppas. Drug diffusion front movement is important in drug release control from swellable matric tablets, *J. Pharm. Sci.* **84**:991–997 (1995).
5. I. S. Moussa, and L. H. Cartilier. Characterization of moving fronts in cross-linked amylose matrices by image analysis, *J. Contr. Rel.* **42**:47–55 (1996).
6. R. Wilding, C. D. Melia, and K. A. Khan. Changes in drug distribution and internal structure of oral pellet systems during in vitro dissolution: cryogenic scanning electron microscopy and X-ray microanalysis, *Pharm. Technol. Int.* **3**:324–329 (1991).
7. am-Ende, M. T. and N. A. Peppas. Analysis of drug distribution in hydrogels using Fourier transform infrared microscopy, *Pharm. Res.* **12**:2030–2035 (1995).
8. L. Pereswetoff-Morath, and P. Edman. Dextran microspheres as a potential nasal drug delivery system for insulin—in vitro and in vivo properties, *Int. J. Pharm.* **124**:37–44 (1995).
9. L. S. Cutts, S. Hibberd, J. Adler, M. C. Davies, and C. D. Melia. Characterizing drug release processes within controlled release dosage forms using the confocal laser scanning microscope, *J. Contr. Rel.* **42**:115–124 (1996).
10. P. Wedekind, U. Kubitscheck, and R. Peters. Scanning microphotolysis: a new photobleaching technique based on fast intensity modulation of a scanned laser beam and confocal imaging, *J. Microsc.* **176**:23–33 (1994).
11. W. N. E. van Dijk-Wolthuis and J. J. Kettenes-van den Bosch, A. van der Kerk-van Hoof, and W. E. Hennink. Reaction of dextran with glycidyl methacrylate: an unexpected transesterification, *Macromolecules* **11**:3411–3413 (1997).
12. R. J. H. Stenekes, O. Franssen, E. M. G. van Bommel, D. J. A. Crommelin, and W. E. Hennink. The preparation of dextran microspheres in an all-aqueous system: effect of the formulation parameters on particle characteristics, *Pharm. Res.* **15**:557–561 (1998).
13. R. Peters and M. Scholz. Fluorescence photobleaching techniques. In R. J. Cherry (ed), *New Techniques of Optical Microscopy and Microspectroscopy*, Macmillan, New York, 1991, pp 199–228.
14. P. Wedekind, U. Kubitscheck, O. Heinrich, and R. Peters. Line scanning microphotolysis for diffraction-limited measurements of lateral diffusion, *Biophys. J.* **71**:1621–1632 (1996).