Chapter 5
Characterization of the mode of incorporation of lipophilic compounds in solid dispersions at the nano-scale using Fluorescence Resonance Energy Transfer (FRET)

D.J. van Drooge[1,*], K. Braeckmans[2,*], W.L.J. Hinrichs[1], K. Remaut[2], S.C. de Smedt[2] and H.W. Frijlink[1]

1: Groningen University Institute of Drug Exploration (GUIDE)
Department of Pharmaceutical Technology and Biopharmacy,
Groningen, The Netherlands
2: Laboratory of General Biochemistry and Physical Pharmacy
Ghent University, Ghent, Belgium
*: equally contributing authors

Submitted

Keywords:
Amorphous clusters, mode of incorporation, fluorescence resonance energy transfer (FRET), solid dispersion, molecular dispersion.

5.1. Summary

Efficient engineering of solid dispersions stagnates by the current inability to establish the mode of drug distribution on a molecular level at a low drug load. This study describes the application of Fluorescence Energy Resonance Transfer (FRET) to characterize the mode of incorporation of dispersed lipophilic molecules in a solid matrix. This method is not limited by microscopic resolution, but detects clustering of drug molecules by proving whether they are in close proximity at the nanometer scale. FRET between a donor and acceptor is only possible within the Förster distance (3-10 nm). In this study, two different lipophilic fluorophores were used as model substances. A donor, Bodipy R6G, and an acceptor, Bodipy650/665, were incorporated in poly(vinyl pyrollidone) (PVP) to form solid dispersions. Two different production processes were used: lyophilization and fusion. The efficiency of the resonance energy transfer from donor to acceptor was measured by confocal microscopy. When the concentration of lipophilic molecules was increased from 0.5 to 1 wt-%, the FRET efficiency increased from 0.25 to 1.31 for solid dispersions prepared by lyophilization and from 0.55 to 1.72 for solid dispersions prepared by a fusion method. The occurrence of FRET in the samples prepared by lyophilization indicates that at least a part of the incorporated molecules is present as clusters. The higher FRET efficiency in the solid dispersions prepared by the fusion method indicates that the fraction of clusters was higher. Furthermore, the results show that the higher FRET efficiency for samples with a higher concentration of lipophilic molecules indicates that the fraction of drug molecules in clusters increases with increasing drug concentrations. The method can be used both qualitatively and semi-quantitatively (comparative). Furthermore, the method can be used to compare solid dispersions of the same composition but prepared in a different way. By choosing donor and acceptor molecules with physicochemical properties close to that of drug to be included, this method is suitable to investigate the effects of changes in the production process or process conditions on the mode of incorporation at the nano-scale of lipophilic molecules in solid dispersions.

5.2. Introduction

To increase dissolution rate limited bioavailability of orally administered poorly soluble drugs, solid dispersions can be used [83]. Solid dispersions consist of a hydrophilic matrix in which lipophilic drug molecules are incorporated. The lipophilic molecules can be dispersed molecularly, incorporated as amorphous clusters or as crystalline particles. This is schematically depicted in figure 1.

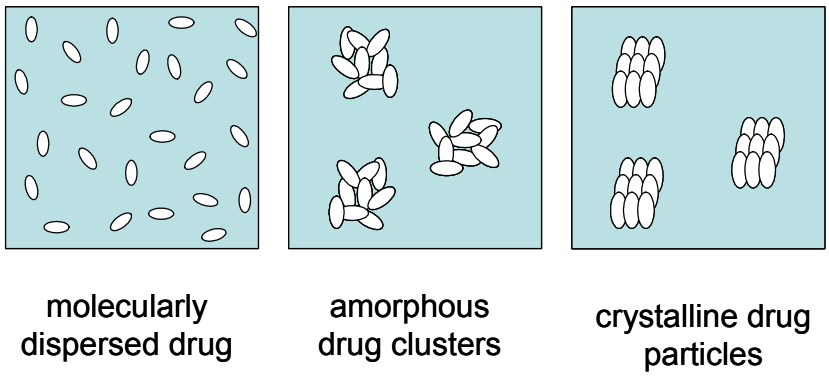

Figure 1: Schematic representation of different modes of incorporation.

Combinations of these modes of incorporation are also possible. The exact knowledge of the mode of incorporation is important because it governs both the stability and dissolution behaviour of the solid dispersion [32, 34]. Therefore, an accurate measurement of the molecular arrangement is a precondition for efficient engineering of solid dispersions [130]. Consequently, such a measurement technique could improve the understanding of new solid dispersions based products and the effect of process conditions, thereby enabling further optimization of the engineering process. Techniques currently available to investigate the mode of incorporation focus on detecting crystallinity rather than detecting amorphous material [33]. Only a few studies have been carried out to assess the discrimination between amorphous drug clusters and a molecularly dispersed drug. For example, confocal raman spectroscopy was used to measure the homogeneity of a solid dispersion of ibuprofen in PVP [63]. However, detailed spatial information from such studies is limited due to the inherent finite optical resolution of the imaging instrument. Alternatively, the restrictions imposed by the limited microscopic resolution can be circumvented by measuring bulk properties of the solid dispersion. A typical example of a bulk property that is related to the mode of incorporation is the glass transition temperature (T_g), which can be determined by differential scanning calorimetry (DSC). The T_g of a solid dispersion with only molecularly incorporated molecules is a function of the composition and the glass transitions temperatures of the pure components. However, when the drug load is low and/or when the difference between the glass transition temperatures of both components is small, the expected shift of the T_g of the pure matrix material may be small and difficult to detect. On the other hand, a solid dispersion containing only amorphous clustered molecules should display two glass transitions, one of the matrix material and one of the lipophilic molecules. However, at low concentrations of lipophilic molecules its T_g may not be detected because the change in specific heat may be too small to be discerned in the thermograms [175,188]. Therefore, due to their limited sensitivity, bulk measurement techniques are not suitable for solid dispersions with a low concentration of lipophilic molecules. Obviously, there is a need for a technique that detects the presence of amorphous drug clusters at low drug loads, typically less than 1 wt-%.

In this study, we propose such a technique based on Fluorescence Resonance Energy Transfer (FRET). For FRET, one needs two types of fluorescent molecules with such properties that after excitation of the first fluorescent molecule, part of the emission energy will be absorbed by the second molecule. The molecules are denominated as donor and acceptor, respectively. When the acceptor is not fluorescent, i.e. a quencher, FRET is only indicated by a decrease in donor emission. When the acceptor is also fluorescent, FRET can be measured by the decrease in donor emission and/or by the increase in acceptor emission [189]. The energy is transferred non-radiatively via long-range dipole-dipole coupling [190]. The efficiency of energy transfer depends on the spectral overlap of donor emission and acceptor absorption spectra, and on the relative orientation of the donor and acceptor molecules. Furthermore, the transfer efficiency strongly depends on the intermolecular distance: it is inversely proportional to the sixth power of the intermolecular distance. FRET can therefore only occur when the donor and acceptor molecules are in close proximity. The distance below which FRET can occur, the Förster distance, depends on the donor-acceptor pair and is typically between 3 and 10 nm [191].

In this study, the lipophilic drug is represented by two different fluorescent lipophilic molecules, which are incorporated in a matrix of PVP. In the extreme case where all molecules are separated from each other by a distance of more than approximately 10nm, no FRET signal will be detected at all. On the other hand, when all molecules are present in clusters, where the intermolecular distance is smaller than 10 nm, the FRET efficiency will be maximal. Between these extremes, a gradient in the FRET signal will be observed. This implies that the FRET efficiency is a measure for the intermolecular distance in the matrix and thereby for the fraction of molecules that are incorporated as separate molecules or as clusters. Consequently, the FRET efficiency provides a qualitative and quantitative indication of the mode of inclusion. However, it should be noted that this technique can only be applied at low concentrations of incorporated molecules, since at higher concentrations, even for molecularly dispersed molecules, the distance between donor and acceptor molecule will be below 10nm anyway.

In this study, it will be investigated whether FRET signals can be measured in solid dispersions with PVP as matrix material. We will outline the experimental method and the calculations for the quantification of the FRET signal from the confocal microscopy images. We also propose a theoretical model that relates the measured FRET intensities to the fraction of clustered molecules. The method will be applied to solid dispersions prepared by two different preparation methods, because it is well-known that the production process can have a large effect on the mode of incorporation [33]. Solid dispersions prepared by lyophilization [174] are compared with samples prepared by the fusion-method [130]. Finally, an outline will be given for future experiments and improvements that will enable the practical application of FRET measurements to quantify the fraction of clustered lipophilic molecules in solid dispersions.

5.3. Theory

The basis of FRET analysis is found in the fact that the fluorescence emission of the donor and acceptor molecules can be detected at different wavelengths. Let D and A denote the fluorescence signal from the donor and acceptor molecules, respectively. After excitation of a donor molecule, either it will emit a fluorescent photon, or, if there is an acceptor molecule within the Förster distance, the energy will be transferred non-radiatively to the acceptor molecule, which will emit a fluorescent photon (of a different wavelength) in its turn. If all molecules are molecularly dispersed, FRET cannot occur. Only fluorescence of the donor will be observed. However, if a fraction of the donor and acceptor molecules is present in clusters, there will be fluorescence from the acceptor molecules as well (at the expense of donor fluorescence). The FRET efficiency can therefore be quantified as the ratio of acceptor to donor fluorescence: $FRET = A/D$. Since the acceptor fluorescence can only arise from clustered acceptor molecules, whereas donor fluorescence can arise from both clustered and molecularly dispersed donor molecules, the FRET efficiency can also be written as:

$$FRET = \frac{A^{cl}}{D^{cl} + D^{mol}} \ , \qquad \text{(Eq. 1)}$$

where the superscripts cl and mol denote the origin of the fluorescence signal: from clustered or molecularly dispersed molecules, respectively.
Let f be the fraction of drug molecules that is located in clusters. Furthermore, let $\overline{D^{cl}}$ and $\overline{D^{mol}}$ denote the donor fluorescence for a sample where all drug molecules are clustered or molecularly dispersed, respectively. In that case, we can write:

$$D^{cl} = f \cdot \overline{D^{cl}} \qquad \text{(Eq. 2)}$$

and

$$D^{mol} = (1 - f) \cdot \overline{D^{mol}} \ . \qquad \text{(Eq. 3)}$$

Next, we assume that the molecular density of the clusters is the same for all clusters in a particular sample. Consequently, the intermolecular distance in clusters is constant, and hence no differences in the FRET efficiency are expected for the clustered molecules. Furthermore, we assume that boundary effects caused by a reduced number of donor-acceptor contacts at the edge of a cluster can be neglected, which implies that the size of a cluster will not affect the FRET efficiency. Finally, we can assume that the donor and acceptor molecules in the clusters are randomly distributed at the same ratio as in the whole sample. This is likely to occur when the physico-chemical properties of donor and acceptor are similar. From this line of reasoning, it follows that the FRET efficiency for a single cluster is the same as the FRET efficiency measured by microscopy in a sample containing only clusters. For such a sample we have:

$$\overline{FRET^{cl}} = \frac{\overline{A^{cl}}}{\overline{D^{cl}}} = \frac{A^{cl}}{D^{cl}} . \qquad \text{(Eq. 4)}$$

By combining Eq. 4.and Eq. 2, we then find:

$$A^{cl} = f \cdot \overline{D^{cl}} \cdot \overline{FRET^{cl}} . \qquad \text{(Eq. 5)}$$

By substituting Eqs. 2, 3 and 5 into Eq. 1 we finally find:

$$\frac{FRET}{\overline{FRET^{cl}}} = \frac{f}{f\left(1 - \frac{\overline{D^{mol}}}{\overline{D^{cl}}}\right) + \frac{\overline{D^{mol}}}{\overline{D^{cl}}}} , \qquad \text{(Eq. 6)}$$

where f is the fraction of clustered drug molecules, $\overline{FRET^{cl}}$ the FRET efficiency for a sample containing only clustered drug molecules ($f = 1$), $\overline{D^{mol}}$ and $\overline{D^{cl}}$ the donor fluorescence for a sample containing only molecularly dispersed or clustered drug molecules, respectively. Eq. 6 can be used to quantify f for a particular solid dispersion by a FRET measurement. The parameters $\overline{FRET^{cl}}$, $\overline{D^{mol}}$ and $\overline{D^{cl}}$ should be determined separately from solid dispersions containing only clustered of molecularly dispersed molecules. It is important to note that such solid dispersions should have the same concentration of donor and acceptor molecules as the sample for which FRET is to be determined. In addition, exactly the same excitation intensity should be used for the measurement of $\overline{D^{mol}}$ and $\overline{D^{cl}}$. Eq. 6 has been plotted in figure 2 for different ratios of $\overline{D^{mol}} \big/ \overline{D^{cl}} = n \quad (n \geq 1)$.

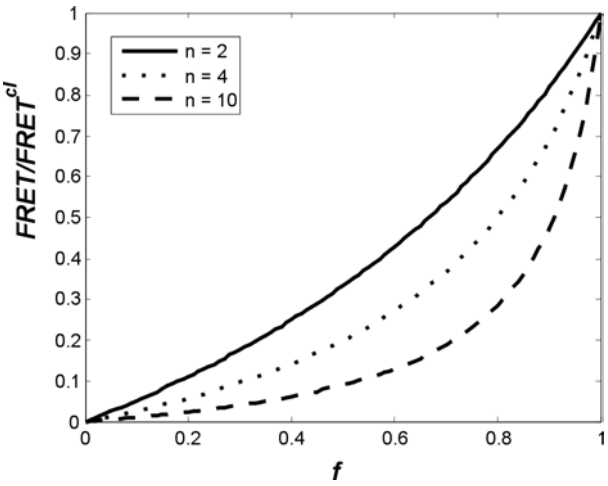

Figure 2: FRET efficiency vs. the fraction f of molecules that is situated in clusters according to Eq. 6 for 3 different values of $\overline{G^{mol}} \big/ \overline{G^{cl}} = n$.

5.4. Materials

Tertiary butanol (TBA) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Polyvinylpyrollidone-K30 (PVP) was provided by BUFA B.V. Uitgeest, The Netherlands. The fluorescent dyes Bodipy R6G (M_w = 427g/mol) and Bodipy 650/665 (M_w = 643g/mol) were purchased from Invitrogen B.V., Breda, The Netherlands. The spectra and molecular formulae of the fluorescent probes are depicted in figure 3. Demineralised water was used in all cases.

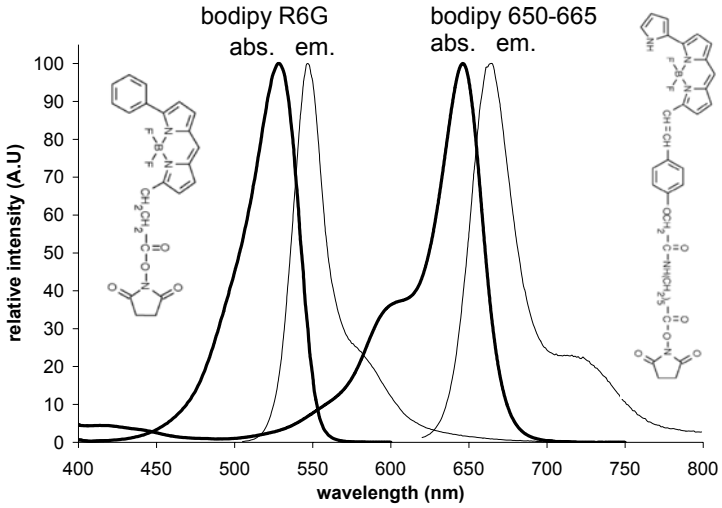

Figure 3: absorption and emission spectra of Bodipy R6G (donor) and Bodipy650/665 (acceptor)

5.5. Methods

5.5.1. Preparation of solid dispersions by lyophilization

The preparation of the solid dispersions was based on a procedure described before [174]. Shortly, the fluorophores and PVP were dissolved in TBA at a total concentration of 5 mg/ml. Different compositions of the solid dispersions were obtained by adjusting both fluorophore and PVP concentrations, while maintaining the total concentration at 5 mg/ml. The solutions were immersed in liquid nitrogen until they were fully frozen. The frozen solutions were lyophilized using a Christ lyophilizer, type Alpha 2-4, (Salm and Kipp, Breukelen, The Netherlands) with a condenser temperature of -53°C. Lyophilization was performed according to a two-step procedure. Firstly, the pressure was set at 0.220 mbar and the shelf temperature at -35°C for one day. Subsequently, the pressure was decreased to 0.05 mbar, while the shelf temperature was gradually raised to 20°C. These conditions were maintained for another day. After removing the samples from the freeze drier,

they were placed in a vacuum desiccator over silica gel at room temperature for at least 1 day.

5.5.2. Preparation of solid dispersions by the fusion-method

Solid dispersions prepared by lyophilization were heated to 200°C in a standard aluminium sample pan using a differential scanning calorimeter (DSC2920, TA Instruments, Ghent, Belgium). The samples were annealed for 10 minutes. Since this is well above the T_g of PVP (171°C), the molecular mobility of PVP was assumed high enough to allow for changes in the mode of incorporation of the fluorophores. Subsequently, the samples were cooled to 20°C and stored in a dry atmosphere.

5.5.3. Microscopy

The solid dispersions were placed on a microscope slide. A cover slip with an adhesive spacer of 0.5 mm thickness (Secure-Seal Spacer; Molecular Probes, Leiden, The Netherlands) was put in between to make sure the solid dispersions were not compressed and deformed between the cover slip and the microscopic slide. Images were recorded with a confocal laser scanning microscope (model MRC1024 UV, Bio-Rad, Hemel Hempstead, UK). A 10× objective lens (CFI Plan Apochromat; Nikon, Badhoevedorp, The Netherlands) with a numerical aperture (NA) of 0.45 was used for imaging. The donor was excited by the 488 nm line from a Kr/Ar laser. The donor and acceptor fluorescence emission were detected in two separate channels. A 540/30nm bandpass filter was used for the detection of the donor emission and a 680/32nm bandpass filter for the acceptor emission. These channels will be referred to as the green and the red channel, respectively. The FRET efficiency was quantified by image processing of the 8-bit confocal images as outlined below.

5.5.4. Calculation of FRET efficiency

The FRET efficiency is quantified as the ratio of fluorescence intensities measured in the green (donor) and red (acceptor) channel. Depending on the optical set-up, the green and red intensity have to be corrected for cross-talk between the channels and direct excitation of the acceptor. In our case, cross-talk involves the (unwanted) detection of donor fluorescence in the red channel. The cross-talk c_D can be quantified in samples containing only donor at the same drug load as in the FRET samples containing both fluorophores: $c_D = R_D / G_D$, where R_D and G_D are the intensities in the red and green channel, respectively. In addition, it is possible that the laser line which is used for excitation of the donor can also excite the acceptor directly in the FRET samples containing both fluorophores. The contribution to the green and red fluorescence signal by direct excitation of the acceptor can be quantified in samples containing only acceptor at the same concentration as for the FRET samples. For the quantification of this correction, it

is important that the laser intensity is exactly the same as during the actual FRET experiment. In this study, the FRET efficiency is therefore calculated from:

$$FRET = \frac{R_{D+A} - R_A - c_D\left(G_{D+A} - G_A\right)}{G_{D+A} - G_A} = \frac{R_{D+A} - R_A}{G_{D+A} - G_A} - c_D \qquad \text{(Eq. 7)}$$

where the subscript A or D refers to samples containing only acceptor or only donor, and the subscript $D+A$ refers to samples containing both fluorophores. Eq. 7 expresses how the FRET efficiency (*FRET* in Eq. 6) can be calculated from the microscopy images.

5.6. Results and Discussion

5.6.1. Donor:acceptor ratio in samples

In preliminary experiments, it was observed that the FRET efficiency of samples with a donor-acceptor mass-ratio of 1:2 was higher than a 1:1 mass ratio, indicating a higher sensitivity obtained with 1:2 mass-ratios. This is most likely because the molar ratio is closer to unity in 1:2-samples, resulting in more donor-acceptor interactions. All further experiments in this study have therefore been carried out with 1:2 donor-acceptor mass-ratios. To avoid quenching and to assure a linear relation between the fluorophore concentration and the observed fluorescence intensity (as determined in a separate experiment), the total concentration of donor and acceptor together was set at 0.5wt-% and 1.0wt-%. For 1:2 mass-ratios, this implies that the donor and acceptor concentrations are 0.167wt-% and 0.33wt-% for the 0.5 wt-% samples or 0.33wt-% and 0.66wt-% for the 1.0 wt-% samples.

5.6.2. FRET efficiency

The FRET efficiency of the different samples can be calculated from the confocal images using Eq. 7. A typical microscopic image is shown in Fig. 4. For each sample, the FRET efficiency has been determined at 3 to 6 randomly chosen regions. Table 1 summarizes the results and reveal the following aspects about the solid dispersions prepared in this study.

Table 1: FRET intensities in the solid dispersion.
The FRET efficiency is calculated as the mean and standard deviations from 3 to 6 individual measurements in each sample.

	FRET efficiency (-)	
Solid dispersions prepared by:	lyophilization	fusion
drug load 0.5wt-%	0.25 ± 0.05	0.55 ± 0.09
drug load 1.0wt-%	1.31 ± 0.21	1.72 ± 0.06

Firstly, it can be noticed that the standard deviations of the FRET efficiencies are relatively small. This indicates that the samples are rather homogeneous on a larger (microscopic) scale. On the other hand, when looking at the values of the FRET efficiencies, the homogeneity on a nano-scale is evaluated. This is a clear difference between a standard microscopic technique and the FRET technique. Secondly, the FRET efficiency is significantly larger than zero, indicating that energy is indeed transferred from donor to acceptor, in spite of the low concentration of fluorescent molecules in these samples. This indicates that nanoscopic clusters of donor and acceptor molecules must exist in which the donor and acceptor molecules are in close proximity.
However, absolute quantification is not possible yet because a small FRET signal can also be detected in case of a molecular distribution, since a completely random distribution of the lipophilic molecules throughout the matrix results in a random distribution of distances between the molecules. Therefore, even at very low concentrations and a random molecular distribution there can be a small fraction of donor and acceptor molecules within the Förster distance giving a small FRET signal. Depending on its magnitude, it might be necessary to take this aspect, which could be quantified from a sample containing only molecularly dispersed molecules, into account for absolute quantification of the FRET efficiencies.

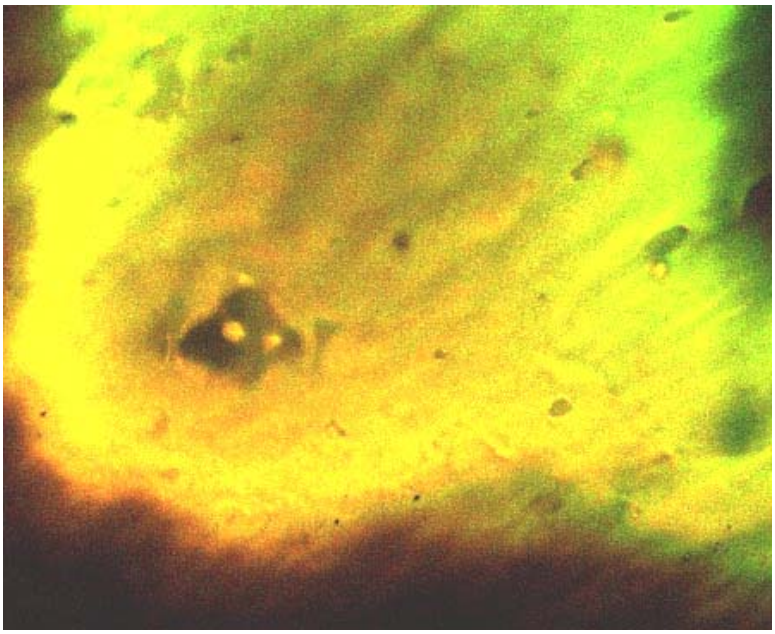

Figure 4: Microscopic image (300 micron x 250 micron) of solid dispersion with 0.5wt-% drug load, i.e. 0.166wt-% donor and 0.33wt-% acceptor, after excitation at 488nm. The emission of the donor is visualised in green and the emission of the acceptor, as a result of FRET, is shown in red. Differences in red and green intensities in the picture give rise to small variations in FRET efficiencies throughout the sample.

We can nevertheless use Eq. 6 for a qualitative interpretation of our first results (see table 1). We observe that for the same drug load, the FRET efficiency for solid dispersions prepared by the fusion-method differs from the FRET efficiency of the lyophilized solid dispersions. Solid dispersions prepared by the fusion-method are obtained by heating a lyophilized solid dispersion in order to bring the PVP above its glass transition and allow for fusion as described in the methods section above. It was observed that the FRET efficiency in these solid dispersions was higher than in solid dispersions prepared by lyophilization. Since both solid dispersions have the same concentrations of incorporated molecules, $\overline{FRET^{cl}}$, $\overline{G^{mol}}$ and $\overline{G^{cl}}$ from Eq. 6 should be identical. According to Eq. 6 (see also figure 2) this indicates that the increase in FRET efficiency must be due to an increased fraction of clustered molecules (*f*) in samples prepared by the fusion-method. Therefore, it can be concluded that during fusion at least a part of molecularly dispersed lipophilic molecules become phase separated, causing an increase in the number and/or size of the clusters. Apparently, heating the matrix does not result in spontaneous mixing of the matrix material with the lipophilic molecules. Instead, they rather seem to form a separate phase as soon as the T_g of PVP is passed allowing the lipophilic molecules to diffuse, promoted by a difference in polarity with PVP. Furthermore, the results in table 1 show a pronounced increase in FRET efficiency (factor 3 to 5) for both kinds of solid dispersions when the concentration of incorporated molecules is increased from 0.5 wt% to 1.0 wt%. This observation implies that the fraction of clustered molecules is higher for higher concentrations of lipophilic molecules. We note that the presence of both molecularly dispersed and clustered drug molecules in the same sample is not uncommon for amorphous solid dispersions as has been reported before [183, 188].

5.7. Conclusions and future outlook

In this study, the proof of concept of a method that gives insight in the structure of solid dispersions at a molecular level (nano-scale) was demonstrated. To our knowledge, this is the first study in which FRET microscopy is applied to solid materials, and in particular to amorphous solid dispersions with relatively low concentrations of incorporated molecules. We have discussed how the experiments can be performed and how the FRET signal can be quantified from the microscopy images by image processing. As a proof of principle, we have used the method to assess the molecular distribution of lipophilic molecules in solid dispersions prepared by lyophilization and prepared by the fusion method. Our first results show that in all solid dispersions investigated in this study, at least a part of the incorporated molecules are located in close proximity of each other (<10nm) which is an indication for the presence of clusters. We have also concluded that solid dispersions prepared by the fusion method contain more clustered molecules due to a more extended phase separation as compared to lyophilized solid dispersions. Furthermore, it was observed that the fraction of clustered molecules increases for higher total drug loads in solid dispersions prepared by the two different methods.

It can be concluded that the current method is suited to investigate the effects of different process conditions on the mode of incorporation of the lipophilic molecules in a solid dispersion both qualitatively and semi-quantitatively.
A straightforward theory is proposed that relates the measured FRET efficiencies to the fraction of clustered drug molecules. To reveal the full quantitative potential of this approach, future research should focus on the preparation of samples containing only clustered drug molecules (f = 1) or only molecularly dispersed molecules (f = 0). From such samples, the parameters $\overline{FRET^{cl}}$, $\overline{D^{mol}}$ and $\overline{D^{cl}}$ from Eq. 6 can be determined.
The two lipophilic fluorophores used in this study could be incorporated in a more lipophilic matrix that is fully miscible with the fluorophores, for example PMMA. This solid dispersion would offer the following advantages. Because there is no tendency for phase separation, no clusters will be formed during preparation of this solid dispersion. The FRET efficiency could be measured as a function of the fusion time. If the FRET efficiency decreases to a plateau value, it can then be assumed that all molecules are molecularly mixed with the matrix. A plateau value larger than zero can consequently be ascribed to random distribution in distances between the fluorophores, but the plateau value will be lower for lower concentrations of incorporated molecules. In this way, a control sample could be obtained with homogeneous distribution of the fluorophores (f = 0). This would enable the measurement of $\overline{G^{mol}}$. Secondly, a more hydrophilic matrix should be chosen to prepare a sample in which the lipophilic incorporated molecules have a high tendency for phase separation. Such samples are also prepared by fusion. In this case, the lipophilic molecules should be completely immiscible with the matrix. Variation of the fusion time again provides an extra control for complete clustering of the lipophilic molecules. Thus, a sample with f = 1 is obtained. In this way, the parameters $\overline{FRET^{cl}}$ and $\overline{G^{cl}}$ are determined, which enables calculation of the fraction of clustered molecules from the FRET efficiency in the solid dispersion of interest.
Finally, tests should be done with the lipophilic drug of interest attached to fluorescent groups to minimize the difference of the physico-chemical properties between the model drug and the real drug. These experiments will show the potential of this new technique to reveal the mode of incorporation of lipophilic molecules in fully amorphous solid dispersions with low drug loads.

5.8. Acknowledgements

Kevin Braeckmans is a Postdoctoral Fellow of the Fund for Scientific Research - Flanders (Belgium).