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Direct and real-time quantification of tenofovir release from pH-sensitive microparticles into simulated biological fluids using ¹H-NMR

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Abstract

In vitro drug release evaluation is a very important step toward the quality control of nano- or micro-particle drug delivery systems. However, most quantitative techniques such as HPLC requires a dialysis membrane to separate the released free drug from these delivery systems, thus are not capable of direct detection and real-time quantification of the drug release. This study describes for the first time, a rapid, specific, and direct method for the real-time quantification of *in vitro* tenofovir (TNF) release from pH-sensitive microparticles using a Varian 400 MHz ¹H nuclear magnetic resonance (¹H-NMR) spectrometer. Various analytical performance parameters such as linearity, precision, accuracy, limit of quantification (LOQ), limit of detection (LOD), and robustness were validated according to International Conference on Harmonization (ICH) guidelines. The *in vitro* release of TNF from microparticles in both simulated vaginal fluid (VFS) and the mixture (VSFS) of VFS and simulated semen fluid (SFS) was monitored and quantified in real-time using ¹H-NMR. The capability of real-time quantification of *in vitro* drug release from microparticles not only provides a more accurate prediction of its biological behavior *in vivo*, but is also independent of potential interference from the dialysis membrane.

Keywords

¹H-NMR; *in vitro* release kinetics; tenofovir; microparticles; analytical method validation; drug delivery system; dialysis; real-time quantification

1. Introduction

In vitro drug release testing is a very important step toward the quality control of drug delivery systems and the prediction of *in vivo* pharmacokinetics¹, especially for nanoformulations given their very small size. The size of these nanoparticles makes it very

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difficult to separate them from release media in a fast and efficient way. So far the membrane diffusion technique has been the most effective and thus widely used method. In this method, the nanoparticle suspension is enclosed in a dialysis bag with appropriate molecular weight cutoff as the donor compartment. It is separated from the receiving compartment where a sink condition for the drug with respect to the donor compartment is maintained. The amount of drug detected in the receiving compartment is generally considered as the amount of drug released from the nanoparticle. Despite its wide acceptance, this method has been criticized as the drug release from nanoparticle itself in the donor compartment does not satisfy the sink condition, therefore the drug release is governed by the partition between two phases separated by the dialysis membrane²⁻⁴. Additionally, Modi et.al. demonstrated that the experimental results of release quantification methods utilizing dialysis can be completely misleading based on their experimental results and simulations.⁵ However, up until 2011, over 40% of the case studies which report drug release profiles from nanoparticles were still using a dialysis method⁶. As demonstrated by the work of Leavy et.al. and Xu et.al.^{7,8}, sometimes a reverse dialysis method is used to compensate for the misinterpretation of release rate, where the nanoformulation is put in the exterior solution and sampling is done from inside the dialysis bag. However, sensitivity and specificity limit the application of this method. Therefore an ideal solution would be a method whose detection and quantification of drug molecule is direct, accurate, and if possible, in a manner that is real-time. Nuclear magnetic resonance spectroscopy (NMR) is an important and commonly used identification tool in analytical method development. NMR can be also used as a quantification tool, due to the fact that the area of integrated signal peak is directly proportional to the number of nuclei contributing to the signal. Since its first application in 1963^{9,10}, quantitative NMR (qNMR) has been widely applied in pharmaceutical and food industry¹¹⁻¹⁴. Some advantages of qNMR have been recognized, such as fast acquisition, non-destructive nature, simple sample preparation and handling, and the ability to measure an analyte in a mixture.¹⁵

Taking advantage of these properties, in this study, a ¹H-NMR method is developed and validated for the first time to perform a real-time detection and quantification of tenofovir (TNF, a nucleotide analog reverse transcriptase inhibitor used as a HIV microbicide) in both simulated vaginal fluid (VFS) and simulated semen fluid (SFS). Tenofovir (TNF, {(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl}oxy} methylphosphonic acid) is a nucleotide analog HIV reverse transcriptase inhibitor whose prodrug (Tenofovir disoproxil fumarate, TDF) is now marketed in an oral dosage form (Viread®, Gilead Science), and its 1% vaginal gel formulation has recently been proven effective in clinical trials.¹⁶ Unfortunately, the current topical gel formulation suffers from disadvantages such as leakage and poor patient compliance which limit its effectiveness and acceptability.¹⁶ Therefore a spray dried mucoadhesive and semen-triggered microspheres (MS) formulation based on polymethacrylate salt has been developed.¹⁷ Methods that can detect and quantify TNF in water or human plasma using different techniques such as high-performance liquid chromatography (HPLC)^{18,19} and liquid chromatography-mass spectrometry (LC-MS)^{20,21} have been reported. However, in order to predict the amount of drug released from this topical formulation in vitro conditions mimicking in vivo scenario, an analytical method capable of directly detecting TNF release in real time in simulated vaginal fluid (VFS) as

well as mixture of vaginal and seminal fluid (VSFS) is necessary. Most of these reported methods are time-consuming, and sometimes require complicated sample preparation and pre-treatment, especially when applied to the detection of drug release from a nanoformulation. For example, in order to detect the amount of TNF released from its nanoparticle formulation, a dialysis membrane is used to exclude any formulation or excipient residue from the release media to avoid compromising the accuracy of drug quantification¹⁸. Our results have demonstrated a good sensitivity as well as specificity, which allows the direct quantification of *in vitro* TNF release from a spray dried mucoadhesive and pH-sensitive microspheres (EuSNa-TNF MS) formulation based on polymethacrylate salt without the membrane diffusion technique.

2. Materials and methods

2.1. Materials

Tenofovir (TNF) was purchased from Zhongshuo Pharmaceutical Co. Ltd. (Beijing, China). Eudragit® S-100 (Methacrylic acid-methyl methacrylate copolymer 1:2) was kindly provided by Evonik Industries (Darmstadt, Germany). Deuterium oxide and sodium hydroxide were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and used without further purification.

2.2. Quantitative NMR (qNMR) theory

qNMR has been proved to be an remarkably successful analytical tool in structural analysis.²² One important fundamental aspect of qNMR is that the signal intensity (I_x) is directly proportional to the number of nuclei (N_x) producing the NMR signal¹⁵.

$$I_x = K_s N_x \quad (1)$$

where K_s is an independent spectrometer constant. Therefore, either the raw integrated signal (I_x) or a ratio of relative integrated peak areas (I_x/I_y) could be used to quantify a molecular concentration. For instance, if multiple ^1H NMR experiments are performed, since K_s is a spectrometer constant across the spectrum, the relative integrated signal area is equivalent to the concentrations. In this paper, the integrated peak area of the protons in TNF was used to indicate the amount of drug.

2.3. ^1H NMR method

^1H NMR spectra were carried out on a Varian (Palo Alto, CA, USA) 400MHz spectrometer with a Varian two channel probe. Vnmrj 2.2 (Palo Alto, CA, USA) was used to process the experiments. A Bruker (Fremont, CA, USA) 250MHz NMR, operated by Tecmeg software 2.3.8-50615 (Houston, TX, USA) was also used for the robustness study. Typically, the spectra were acquired with a 90° pulse length which was $14.2 \mu\text{s}$. The relaxation delay was 2 sec and the number of scans was 128. The T_1 of the target protons of TNF were 2 sec and 8 sec which typically requires up to 40 sec ($5 \times T_1$) for relaxation. However, since the experimental parameters were fixed between all spectra (especially the number of experimental scans), the effects of too short a relaxation delay were constant in all experiments making the integral calculation consistent. The primary reason to apply a short

relaxation delay was that acquisition time for each experiment during the *in vitro* drug release study was limited. Shimming was applied to every sample in order to obtain a similar linewidth in each spectrum. Baseline correction, phase adjustment and integral calculation were done manually using MestReNova Lite 5.2.5-4731 (Escondido, CA, USA) software and Matlab 6.1 (Natick, MA, USA). Each experiment was performed in triplicate. Kinetics experiments were applied for quantification of TNF released from microsphere. 70 spectra were measured in one kinetics experiment and the time interval between two measurements was 3.3 min. The total time for one release experiment was about 4 hours.

2.4. Preparation of standard and sample solutions of TNF

Simulated vaginal fluid (VFS, pH 4.2) and simulated semen fluid (SFS, pH 7.6) were prepared individually according to published method^{23,24}, with the exception that D₂O was used instead of water. Briefly, the VFS was prepared using sodium chloride, potassium hydroxide, calcium hydroxide, albumin bovine fraction V, lactic acid, glycerol, urea, acetic acid, glucose and D₂O. The components of SFS were: sodium phosphate monobasic/dibasic, sodium citrate, potassium chloride, potassium hydroxide, fructose, glucose, lactic acid, urea, and bovine serum albumin. Owen et. al. reported that the volume of normal human vaginal fluid is 0.75 mL, and the volume of human ejaculate is 3.4 mL.^{23,24} Therefore VFS and SFS mixture (VSFS, pH 7.6) at 1:3 ratio was prepared to mimic the scenario of human ejaculate presence. The standard stock solution of TNF (50 mM) was prepared by dissolving 143.5 mg of TNF in 10 mL of VFS and VSFS. Serial dilutions of stock solution with the same diluents led to the sample solutions in the concentration range of 0.2-5 mM for ¹H-NMR analysis.

2.5. Method validation

Five different samples with TNF concentrations from 0.2-5 mM were selected to construct the standard curves of the method. Four concentrations (0.5, 1, 2, 5 mM) were utilized to validate the method according to International Conference of Harmonization (ICH Q2:R1) guidelines²⁵. The variation within the ¹H-NMR peak areas were presented as relative standard error (% RSE) for each validation parameters.

2.5.1 Linearity—Linearity represents the linear relationship between the test results and the concentration of the analyte within a certain range. In this study, a calibration curve was prepared by analyzing the relative peak areas of TNF compared to the residual water peak. The intercept, slope, and squared correlation coefficient (R^2) were determined by linear regression analysis.

2.5.2 Specificity—The specificity of the method was determined by analyzing the potential interfering peaks from formulation/media excipients. The ¹H-NMR spectrum of standard TNF solution in VFS/VSFS was compared with that of pure VFS/VSFS as well as TNF loaded microspheres/blank microspheres suspended in both media to ensure specificity.

2.5.3 Limit of detection and limit of quantification—The limit of detection (LOD) and limit of quantification (LOQ) were defined as the concentrations that could yield a signal-to-noise ratio of 3:1 and 10:1, respectively.²⁵

2.5.4 Accuracy and precision—Accuracy of the method was assessed using four concentration levels (0.5, 1, 2, 5 mM) and reported as percent mean recovery calculated from each concentration using the calibration curve. Each concentration was analyzed in triplicate. Precision was evaluated as intra-day precision as well as inter-day precision using the above concentration levels as in accuracy validation. Each concentration was analyzed in triplicate and the data was represented as the percentage of Relative Standard Error (%RSE).

2.5.5 Robustness—The robustness of the method was evaluated by analyzing three parameters which were magnetic field, relaxation delay and 90° pulse length. The levels of these robustness parameters are shown in **Table 1**. Each measurement was performed in triplicate and the data was represented as %RSE.

2.6 Preparation and loading efficiency of EuSNa-TNF microspheres

EuSNa-TNF MS was prepared by a published method.¹⁷ Briefly, different amounts of S-100 and TNF were added in 20 mL of deionized water with an appropriate amount of NaOH to achieve complete salification. The solution was then spray dried using a Buchi Mini Spray Dryer, Model 290 (Buchi Laboratories - Technik AG, Flawil, Switzerland). The dried microspheres were weighed using a Mettler Toledo XS 105 dual range balance (Mettler Toledo Inc., Columbus, USA), and stored in a sealed or capped glass container at 4 °C. The physicochemical properties of these microspheres, including drug loading efficiency (4%), were also characterized in the above publication.

2.7 Real-time *in vitro* release study

To test the TNF release from EuSNa-MS, 12 mg of EuSNa-TNF MS with TNF loading of 4% (w/w) was added into 0.5 mL of VFS (vaginal fluid simulant, pH=4.2) and mixture of VFS and SFS (seminal fluid simulant, pH=7.6) with ratio 1:3 (VSFS), respectively. The samples were prepared in a 5 mm NMR tube and directly analyzed using ¹H-NMR at 37 °C with spinning speed 20 kHz. ¹H-NMR Spectra were measured in series and 70 spectra were collected after 4 hours release.

2.8 *in vitro* release study of TNF using dialysis method

To test our hypothesis of potential interference of the dialysis membrane in quantifying drug release, 1 mL of pure TNF solution was added into a dialysis tube (Spectra/Por Float-A-Lyzer G2, MWCO 3.5-5 KD, Spectrum Laboratories Inc. Rancho Dominguez, CA, USA), and maintained in 30 mL dialysis medium with a shaking speed of 60 rpm at 37 °C. Three dialysis media were tested: D₂O, VFS medium and VSFS medium. The concentration of TNF in the receiving compartment was measured by ¹H-NMR at predetermined time intervals (15 min, 30 min, 60 min, 120 min and 240 min). One control sample was prepared using the same concentration of TNF in the donor compartment. Each control sample was diluted with dialysis medium 1 : 30 in order to simulate the maximum expected concentration after dialysis assuming complete equilibration between the donor and receiver compartments. The concentration of TNF in the diluted control sample was also measured by ¹H-NMR.

3. Results and Discussion

In this study, integrations of the two aromatic proton peaks of TNF were used to quantify the amount of TNF in both VFS and VSFS media shown in **Fig.1**. The linewidth (**Eqn.2**) of NMR signal directly depends on T_2 (spin-spin relaxation time) which varies from different molecules. T_2 is usually very small (around 10^{-5} s) for large molecules such as polymers or proteins, which gives rise to very broad peaks. Therefore, if TNF was free in the solution, the proton signals could be easily detected. However, if TNF was encapsulated inside the polymer, the peak generated from TNF will be very broad because of the slow tumbling.

$$linewidth = \frac{1}{T_2} \quad (2)$$

In practice, the proton peaks of TNF encapsulated inside the polymer were too broad to be seen and only the narrow and sharp peaks generated from free TNF were visible and quantifiable.

3.1 Linearity

To quantify the amount of TNF in both *in vitro* release media, the peak areas of five different concentrations of TNF were plotted vs. their specific concentrations using the linear least-squares method as shown in **Figs. 2** and **3**. The linear equations are $y = 0.5239x + 0.0029$ (in VFS, **Fig. 2**) and $y = 0.614x - 0.0012$ (in VSFS, **Fig. 3**). The R^2 is 0.9998 in VFS and 0.9998 in VSFS.

3.2 Specificity

Specificity is a typically a required precondition for quantitative NMR and is always tested prior to other validation parameters. NMR spectra of pure TNF (2 mM), pure S-100, VFS, VSFS, partially released EuSNa-TNF MS/VFS (0.15 mM) and partially released EuSNa-TNF MS/VSFS (0.92 mM) were analyzed to demonstrate the lack of interference peaks between the signals of interest in TNF and any other signals from the other components of each sample (see **Fig.4**). The protons of interest in TNF ($\delta=8.00$ and 8.09 ppm) are independent from the protons of all other ingredients, establishing specificity, making it possible to quantify the amount of TNF by calculating the peak area of these two peaks. The specificity requirement is the most significant limitation on utilizing or generalizing this reported method for the analysis of other drugs encapsulated into other type of nanoformulations. However, once specificity is established for a given drug/formulation/solvent combination, the method should be applicable. Additionally, it is noteworthy that many drugs are reasonably expected to achieve this specificity considering that a large number of low MW drugs contain aromatic protons which have chemical shift around 8.0 ppm (similar to TNF) which is easily resolved from typical proton signals for solvent around 1.0-6.0 ppm.

3.3 Limit of detection and limit of quantification

The LOD values of the proposed method are 0.0005 mM (143.5 ng/ml) in VFS and 0.005 mM (1435 ng/ml) in VSFS, and the LOQ values are 0.005 mM (1435 ng/ml) in VFS and

0.05 mM (14.35 µg/ml) in VSFS, respectively. ¹H-NMR has relatively lower sensitivity compared to other analytical methods such as HPLC and LC/MS. For example, it was found that the LOD and LOQ values of tenofovir in water, when detected by HPLC, were 50 and 100 ng/ml, respectively¹⁸. The LOQ values typically vary from 3 ng/ml to 20 ng/ml in LC-MS methods^{20,21,26}. However, the levels of LOD and LOQ values found in this study are comparable with other published applications of NMR in the field of pharmaceutical research^{27,28}, including those using a higher magnetic field^{29,30}. Although LOD and LOQ values may be lower using other methods (i.e. the HPLC method), the focus of this study is to quantify the amount of TNF release in biological fluid simulant rather than determine its aqueous solubility limit. Therefore it is reasonably speculated that NMR derived LOD and LOQ values may frequently be sufficient to establish *in vitro* drug release in the ~µg quantities that are typical of drug formulations, as in this study.

3.4 Accuracy and precision

According to ICH guidelines, accuracy is used to study the closeness of agreement between values in a specific analytical method²⁵. In this study, the accuracy was evaluated as mean recovery of a known concentration of the drug. The results are shown in **Table 2**. The method is found to be accurate, as the mean percent recovery is found to be within the range of 90%-110% with all %RSE values being less than 2%, which is acceptable according to ICH guidelines. The precision of the method is demonstrated in **Table 3** and **4**, as all RSE values within a given day and among three different days are less than 2%. The range of precision of published quantitative ¹H-NMR methods could be found from 3% to 8%^{31,32}, and the precision data of TNF quantitative NMR method is comparable to those of the HPLC method¹⁸.

3.5 Robustness

Testing of robustness has always been a critical step in method validation of an analytical method. In the work published by Malz et.al.¹⁵, a variety of NMR instrumental parameters were screened for their effect on method robustness, including pulse power for 90°, preacquisition delay, acquisition time, relaxation delay, etc. It has been found in that study parameters such as relaxation delay and acquisition time (90° pulse length) can affect the signal to noise ratio as well as signal intensity. The change in magnetic field of NMR will have an impact on both signal to noise ratio as well as the sensitivity of the method²⁷. Given the above considerations, those three parameters were chosen in the robustness assessment. As shown in **Table 1**, a variation in those parameters does not significantly affect the outcome of the method

3.6 Real-time *in vitro* release study

The *in vitro* release profile of TNF is found to be different in VFS than in VSFS as shown in **Fig. 5**. More than 40% of TNF was released from EuSNa MS at pH 7.6 (VSFS) in the first 20 min, whereas only 10% was released at pH 4.2 (VFS). After 4 hours, EuSNa MS released about 90% of its payload in VSFS but only about 30% in VFS. This result is supported by the fact that TNF is encapsulated in a pH sensitive polymer (Eudragit® S-100) which is soluble when pH is above 7. A similar trend in the release rate was also observed by our

collaborator¹⁷. Compared with the traditional quantification methods such as dialysis membrane-HPLC, this ¹H-NMR method has several merits: (1) The release sample was able to be kept in NMR during the whole release period, even while quantification occurs. This allows for more data points to be collected, which essentially could be considered as a real-time quantification method. Instead of quantifying the release at several time points (usually less than 10), the entire release profile can be monitored based on periodic measurements (70 spectra for each release study). This provides a more accurate measurement of release kinetics that is not achieved by current methods. (2) Since this method does not require a dialysis membrane, it eliminates the possibility of interactions between drug molecules and the dialysis membrane, which has been suggested in previous cases.^{2,6} TNF is a small hydrophilic molecule drug. Therefore it has been reasonably speculated that a dialysis membrane would not interfere with its release as suggested by comparison with a HPLC method¹⁵, however this is not the case, as seen below. This method will likely also prove amenable to hydrophobic or somewhat larger MW drug molecules that are more likely to experience membrane interactions (once specificity is established). (3) Sample handling and preparation is simple. Typically, samples are prepared in NMR tubes and detected directly. During release, all the measurements will be automatically done without transferring the samples or setting up the experiments.

3.7 in vitro release study of TNF using dialysis method

In order to simplify the dialysis method and to rule out the possible effect from VFS or VSFS medium, pure D₂O was used as a dialysis medium. The control sample was diluted 30 times in order to achieve the projected concentration after complete dialysis. In theory, if there was equilibration of TNF between the donor and receiving compartments, both control and long-time test samples should have the same concentration. NMR spectra were taken for the diluted control sample and the test samples after 4 hours of dialysis. The concentration of TNF was 0.19 mM in the diluted control sample in D₂O, which is the final concentration of TNF assuming no interaction with dialysis membrane. However, no TNF signal was detected after 240 min in the D₂O test samples from receiving compartment. Similar results were observed in VFS and VSFS medium. The concentration of TNF in diluted control sample was 0.21 mM, while the concentration of TNF was 0.14 mM after 4 hours dialysis in VFS medium. For VSFS medium, the expected concentration of TNF was 0.08 mM at equilibrium but no TNF signal was detected after 4 hours in the receiving compartment. Therefore it is reasonably speculated that: (1) free TNF did not achieve equilibrium during the release period. Therefore, the data obtained for a drug release using the dialysis method will not be accurate if the formulation has a fast release time (<~100s min) because, the amount of drug measured in the receiving compartment will be significantly underestimated. (2) More TNF was obtained in the VFS receiving compartment compared with D₂O or VSFS, which indicated the specific dialysis medium likely also has an effect on the TNF partitioning. One main difference between VFS and D₂O/VSFS media was the pH. In our results, lower pH showed a higher degree of TNF partitioning to the receiving compartment than higher pH. Other groups have also reported similar results for different systems as we obtained here. For example, Modi et.al. demonstrated that utilizing dialysis methods to perform release quantification can result in data that is completely misleading based on their experimental results and simulations.⁵ They suggested appropriate control experiments and

mathematical treatment of interpreting the data were necessary to determine drug release correctly using dialysis methods. Additionally, Zambito et.al. reported on the reliability of drug release from nanoparticles studies using dialysis methods and their results indicated the release rate was controlled by the transport rate across the dialysis membrane instead of the release rate from nanoparticles.⁶ Therefore, real-time drug release evaluation using ¹H NMR is likely more accurate compared to a method that requires dialysis. Although NMR may have drawbacks, such as a lower LOD and LOQ, it seems to be a more reliable method in this specific application. Additionally, this NMR method perform a real-time quantification of drug release, and it is simple and straightforward.

4. Conclusions

In this study, a real-time quantitative ¹H NMR method was demonstrated to be a simple, rapid and precise way to quantify TNF release from an encapsulated microparticle formulation. This method was validated according to the International Conference on Harmonization (ICH) guidelines, and was found to have a good linearity up to 5 mM, to be precise and robust, and accurate. Good specificity and sensitivity (LOD,LOQ) were also established. It is anticipated that this method will be applicable to a large number of important drug release studies *in vitro*, though these two conditions may not always be achievable depending on spectral overlap and drug solubility,

The *in vitro* release profile of TNF loaded EuSNa MS was established using this direct quantitative method. Real-time quantification of TNF release has demonstrated the significant difference in release rate of EuSNa MS when in biological fluids with different pH. The advantages of this method such as real-time quantification, ease of preparation, and direct measurement without a dialysis membrane have demonstrated it is a promising alternative in the study of *in vitro* drug release from an encapsulated nanoparticle formulation.

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Fig. 1.

¹H NMR spectrum of 5mM TNF in D₂O. The two peaks ($\delta=8.00$ ppm and $\delta=8.09$ ppm) were generated from the aromatic protons in TNF. The integration of these two peaks were used to quantify TNF. The peak ($\delta=4.61$ ppm) was generated from protons in water. The peaks with chemical shift range 3-4.5 were generated from protons 3,4 and 5. The peak at $\delta=0.9$ was generated from a methyl group. The reason choosing the two aromatic peaks for quantification is due to specificity. Please see section 3.2.

Fig. 2.

Standard curve of TNF in VFS. The linear equation is $y = 0.5239x + 0.0029$ and R^2 is 0.9998. This curve was used to interpret the amount of TNF released from microparticles in VFS.

Fig. 3.

Standard curve of TNF in VSFS. The linear equation is $y = 0.614x - 0.0012$ and R^2 is 0.9998. This curve was used to interpret the amount of TNF released from microparticles in VSFS.

Fig. 4.

¹H NMR spectra of (a) pure TNF; (b) S-100; (c) VSFS media only; (d) VFS media only; (e) Partially released EuSNa-TNF MS in VSFS media; (f) Partially released EuSNa-TNF MS in VFS media. The two peaks ($\delta=8.00$ ppm and $\delta=8.09$ ppm) generated from TNF (a,e and f) are specific. No proton signals overlaps with these two peaks in all other molecules (b, c and d). This property makes it possible to quantify TNF by calculating the intensity of these two peaks.

Fig. 5.

In vitro release profile of TNF loaded EuSNa MS in VFS (red curve) and VSFS (blue curve). A series of ^1H -NMR spectra were obtained every 3.3 min for each sample. Seventy data points were collected and used to predict the release curve in both media.

TNF releases more quickly in VSFS than in VFS, since EuSNa MS is pH sensitive which has better resolvability at higher pH level. The percent release at time 0 assumes to be zero in order to simplify the profile.

Table 1Robustness analysis by ^1H -NMR method (n=3)

Robustness parameters	Relaxation delay (s)			90° pulse length (us)			Magnetic field (MHZ)	
Levels	0.98	1.00	1.02	13.9	14.2	14.5	250	400
% RSE (VSFS)	0.684	0.897	0.557	0.799	0.897	0.811	1.080	0.220
% RSE (VFS)	1.870	1.170	1.280	0.332	1.170	1.510	1.506	0.673

Table 2

Accuracy (percent mean recovery) of different levels of TNF (n=3)

Standard concentration (mM)	Recovered concentration (mM)	VSFS Mean recovery $\pm$ SE (%)	RSE (%)	Recovered concentration (mM)	VSFS Mean recovery $\pm$ SE (%)	RSE (%)
0.5	0.507	102.32 ± 0.54	0.53	0.495	100.12 ± 0.57	0.57
	0.517			0.505		
	0.511			0.501		
1	0.976	97.60 ± 0.05	0.05	1.025	102.14 ± 0.21	0.20
	0.977			1.018		
	0.975			1.021		
2	2.040	101.93 ± 0.11	0.10	1.909	95.23 ± 0.13	0.13
	2.041			1.903		
	2.034			1.901		
5	4.956	99.78 ± 0.46	0.46	5.021	100.64 ± 0.12	0.12
	4.978			5.031		
	5.033			5.042		

Table 3Precision of the ^1H -NMR method for quantification of TNF in VFS (n=3).

Concentration (mM)	Intra-day Within-day		Day 1		Inter-day Day 2		Day 3	
	Peak area	%RSE	Peak area ratio	%RSE	Peak area ratio	%RSE	Peak area ratio	%RSE
0.5	0.278	1.534	0.278	1.534	0.274	0.628	0.297	0.694
	0.293		0.293		0.280		0.295	
	0.282		0.282		0.277		0.290	
1	0.546	0.576	0.546	0.576	0.598	0.218	0.660	1.630
	0.538		0.538		0.594		0.665	
	0.549		0.549		0.596		0.629	
2	1.152	0.510	1.152	0.510	1.140	0.129	1.200	0.232
	1.165		1.165		1.136		1.210	
	1.172		1.172		1.135		1.207	
5	3.177	0.327	3.177	0.327	3.047	0.121	3.047	0.697
	3.199		3.199		3.054		3.061	
	3.213		3.213		3.060		3.117	

Table 4Precision of the ^1H -NMR method for quantification of TNF in VSFS (n=3).

Concentration (mM)	Intra-day Within-day		Day 1		Inter-day Day 2		Day 3	
	Peak area ratio	%RSE	Peak area ratio	%RSE	Peak area ratio	%RSE	Peak area ratio	%RSE
0.5	0.262	0.560	0.262	0.560	0.257	1.500	0.268	0.536
	0.266		0.266		0.270		0.274	
	0.267		0.267		0.261		0.271	
1	0.535	0.588	0.535	0.588	0.515	0.277	0.514	0.051
	0.531		0.531		0.512		0.515	
	0.541		0.541		0.510		0.514	
2	1.117	0.747	1.117	0.747	1.071	0.844	1.072	0.107
	1.135		1.135		1.093		1.072	
	1.146		1.146		1.103		1.069	
5	2.645	0.121	2.645	0.121	2.547	0.104	2.600	0.460
	2.644		2.644		2.539		2.611	
	2.635		2.635		2.540		2.640	