



Real-time electrochemical monitoring of drug release from therapeutic nanoparticles

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ARTICLE INFO

Article history:

Received 8 May 2009

Accepted 2 August 2009

Available online 11 August 2009

Keywords:

Nanomedicine

Drug release

Real-time monitoring

Voltammetry

Liposome

Nanoparticles

Doxorubicin

ABSTRACT

An electrochemical protocol for real-time monitoring of drug release kinetics from therapeutic nanoparticles (NPs) is described. The method is illustrated for repetitive square-wave voltammetric measurements of the reduction of doxorubicin released from liposomes at a glassy-carbon electrode. Such operation couples high sensitivity down to 20 nM doxorubicin with high speed and stability. It can thus monitor in real time the drug release from NP carriers, including continuous measurements in diluted serum. Such direct and continuous monitoring of the drug release kinetics from therapeutic NPs holds great promise for designing new drug delivery NPs with optimal drug release properties. These NPs can potentially be used to deliver many novel compounds such as marine-life derived drugs and hydrophobic drugs with limited water solubility that are usually difficult to be characterized by traditional analytical tools.

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1. Introduction

Nanoparticle (NP)-based drug delivery has attracted tremendous attention from both academic and industrial investigators in the past two decades because of its many favorable characteristics [1,2]. It improves the solubility of poorly water-soluble drugs, prolongs *in vivo* drug circulation half-life, reduces the frequency of administration by releasing drugs in a sustained manner, and minimizes adverse systemic effects by delivering drugs preferentially to the target tissues. As a result, numerous NP platforms have been developed or proposed for drug delivery applications including, for example, liposomes, solid lipid NPs, polymeric NPs, dendrimers, silica NPs, and nanoemulsions [3,4]. For all of these therapeutic NPs, their drug release kinetics is a key factor that determines their therapeutic index and potential for clinical use [2,5]. Drug release kinetics represents how fast the drug molecules are released from the therapeutic NPs. Such a release profile is commonly plotted as the weight ratio of the cumulative released drugs to the total drug payload over time [6]. Direct real-time measurements are highly desirable for obtaining reliable assessment of the drug release kinetics.

While several analytical techniques have been employed to quantify the amount of drugs released from therapeutic NPs [7–10], none of these offers a direct continuous monitoring capability. High performance liquid chromatography (HPLC) holds the most popular-

ity in quantifying drugs by directly reading their characteristic UV absorbance upon elution from a proper HPLC column [11]. HPLC thus lacks the real-time monitoring capability and suffers from high procurement and operational costs, lengthy training required, excessive downtimes, and lack of a universal sensitive detector [12]. A fluorometer or scintillation counter can also quantify drugs by reading the fluorescence emission or the ionizing radiation of drug molecules, respectively, if they are pre-labeled with a fluorescent or radioisotope tag. However, tagging drug molecules may alter their diffusion rate and release kinetics.

To measure drug release kinetics, therapeutic NPs are commonly loaded into a dialysis device with a molecular weight cut-off larger than the size of the drug molecules [6,13]. Then the NPs are dialyzed against PBS buffer continuously. The released drugs diffuse out of the dialysis device, driven by osmotic pressure between the two sides of the dialysis membrane. At selected time intervals, a small volume of the dialysis solution is collected to quantify the drugs released from the NPs using one of the techniques mentioned above. Alternatively, one can also collect an aliquot of the NP suspension inside the dialysis device and subsequently break down the NPs to quantify the drugs remaining in the NPs [13]. While these techniques are capable of quantifying drug loading yield and release profile, they usually involve complex procedures and require labor-intensive sample preparation. In addition, before measurements the drugs have to be separated from the NPs by using a dialysis membrane or a centrifugal machine. These can negatively affect the accuracy of the measurements because drugs can adsorb to the dialysis membranes or the high centrifugal force may induce additional drug release from the

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NPs [14]. More importantly, none of these techniques can monitor directly the real-time drug release, as desired for obtaining the most accurate drug release kinetics profile. The sample preparation time for the various techniques also prohibits repetitive measurements at short time intervals. Therefore, it is highly desirable to develop new methods that monitor directly and continuously the drug release kinetics from therapeutic NPs while concurrently simplifying the process and minimizing the errors and costs incurred in the collection and handling of individual sample aliquots.

In this study, we describe an effective electrochemical protocol for direct real-time monitoring of drug release kinetics from therapeutic NPs. Electrochemical devices offer a fast return of the chemical information and have been widely used for continuous (industrial and environmental) monitoring applications [15]. For example, electrochemical sensors, such as pH- and oxygen electrodes, have been used routinely for several decades for real-time measurements. Continuous electrochemical *in vivo* monitoring of drug concentration has also been reported but not in connection to therapeutic NPs [16]. As compared to the fluorescence-based techniques or HPLC described above, the electrochemical route has the potential to directly measure the concentration of free drugs without the need to separate them from the drug-loaded NPs using dialysis membrane or centrifugal machine. Surprisingly, very little research has been carried out to employ electrochemical techniques to monitor drug release kinetics from therapeutic NPs in a real-time manner. In an important study, Tan and Tam [14] have reported the application of a potentiometric drug selective electrode to monitor real-time drug (procaine hydrochloride) release from pH-responsive microgels.

The new electrochemical protocol is illustrated below for the release of doxorubicin from liposomes in connection to repetitive square-wave voltammetric monitoring of the reduction current of the released drug. Square-wave voltammetry is advantageous over potentiometric sensing and represents an attractive technique for monitoring the drug release kinetics with high temporal resolution, as it couples high sensitivity down to nanomolar (nM) range with high speed and reproducibility [17]. The anthracycline doxorubicin has found a wide clinical use in anticancer therapy [18]. The doxorubicin molecule contains quinone and hydroquinone moieties which can be readily oxidized and reduced at various electrode materials [19,20]. For example, a carbon-fiber electrode was used for electrochemical monitoring of doxorubicin efflux from drug-resistant cancer cells [21]. The optimization and characterization of the new voltammetric protocol for direct monitoring of the doxorubicin release kinetics from liposomes are reported in the following sections. The new real-time monitoring capability holds great promise for designing new drug delivery NPs with optimal drug release properties in connection to a wide range of electroactive drugs.

2. Materials and methods

2.1. Reagents and apparatus

Doxorubicin hydrochloride ($2 \text{ mg} \cdot \text{ml}^{-1}$ solution) was obtained from Sigma Aldrich and DOXIL (doxorubicin HCl liposome injection) was from Ortho Biotech Products. Solutions of different concentrations of doxorubicin were prepared in 1X PBS buffer (from GIBCO). The pH value of the solutions were adjusted to 7.4, 6.4 and 5.2, respectively using HCl. Human serum was obtained from Bio-ChemMed Inc. (Winchester, VA). All voltammetric experiments were done using a μ Autolab Type II Analyzer with GPSE software, Eco-Chemie. The 5 mL electrochemical cell consisted of a 2 mm diameter glassy-carbon working electrode, a 1 mm diameter Pt wire counter electrode, and an Ag/AgCl reference electrode (CH Instruments, Austin, TX) inserted through a Teflon cover as illustrated in Fig. 1.

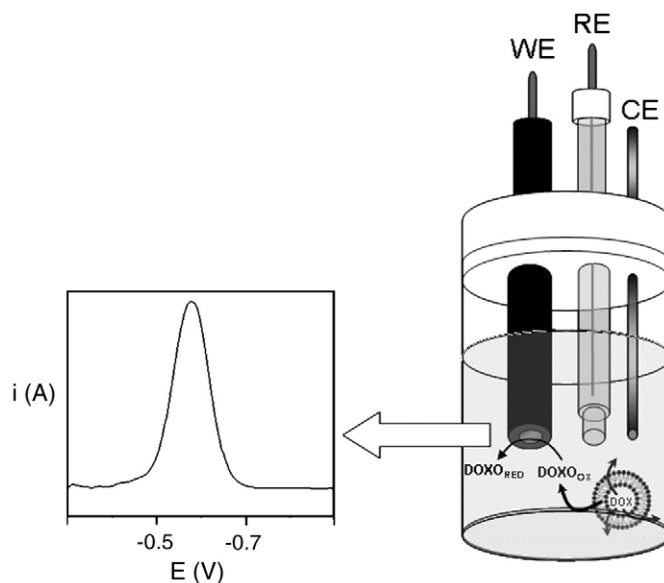


Fig. 1. (Right panel) Schematic illustration of electrochemical cell. Working electrode: glassy-carbon electrode (GCE); reference electrode: Ag/AgCl electrode; and counter electrode: Pt wire. The cell is closed with a Teflon cover and the solution is bubbled with nitrogen. (Left panel) A typical voltammogram of doxorubicin released from the liposomes at pH 7.4.

2.2. Procedure

Nitrogen-saturated solutions (1 ml), used for eliminating oxygen-background contributions, were obtained by bubbling high purity N_2 in the solution for 5 min and continuing with a gentle gas purge (inside the solution) during the voltammetric measurements. The carbon working electrode was initially polished with 3 μm and 0.05 μm alumina slurries, followed by rinsing with water and a 3 min sonication. This mechanical cleaning procedure was performed only once, before a series of runs. Between successive voltammetric runs the electrode was 'cleaned' *in situ* by applying a negative potential (-1.0 V) for 40 s. Then, the electrode was kept at open circuit for 20 s with moderate stirring for providing a short preconcentration period. Square-wave voltammograms of the released doxorubicin were recorded over the -0.3 V to -0.9 V range.

3. Results and discussion

3.1. Optimization and analytical performance

Both the reduction and oxidation of doxorubicin can be used for its electrochemical monitoring [19,20]. Accordingly, we evaluated both the cathodic and anodic voltammetric measurements of the drug. The reduction process of the 5,12-diquinone groups in the doxorubicin molecule was selected for subsequent real-time drug monitoring work because it offers a better reproducibility and allows an *in situ* 'cleaning' of the surface (compared to the manual polishing required between successive anodic measurements of the drug oxidation). Various voltammetric techniques including lineal sweep voltammetry (LSV), differential pulse voltammetry (DPV) and square-wave voltammetry (SWV) were compared. Most favorable conditions, in terms of sensitivity and speed, were observed using the SWV technique. Optimized SWV parameters included a potential scan between -0.3 V and -0.9 V , a frequency of 25 Hz, an amplitude of 25 mV and a step of 2 mV. The measurement system thus consisted of a small volume electrochemical cell (Fig. 1), containing a glassy-carbon disk working electrode (along with an Ag/AgCl reference and a Pt wire counter electrode). A short cleaning step (at a negative

potential) ensures that the adsorbed doxorubicin is removed from the surface, hence erasing potential carry-over memory effects.

The optimal system offers convenient and reliable measurements of trace levels of doxorubicin. Fig. 2 displays square-wave voltammograms for increasing levels of doxorubicin over the 0.1 to 1.0 μM range (Fig. 2 A, a–f). Well-defined voltammetric peaks are observed ($E_p = -0.56\text{ V}$) along with a flat background current. Such favorable signal-to-background characteristics offer convenient measurements of these sub-micromolar concentrations. A low detection limit of around 20 nM doxorubicin can be estimated based on the favorable response to the 0.1 μM doxorubicin solution (Fig. 2 A). Such low detection limit reflects the adsorption-induced signal enhancement (associated with the short preconcentration step) and the effective background discrimination of the square-wave voltammetric operation. The peak intensity increases proportionally with the doxorubicin concentration over the entire range examined. The corresponding calibration plot (Fig. 2 B) is highly linear, with a correlation coefficient of 0.999 (sensitivity of 2.40 $\mu\text{A}/\mu\text{M}$).

The high sensitivity of the voltammetric protocol is coupled with high reproducibility and stability, essential for continuous monitoring of the drug release kinetics. The stability of the doxorubicin reduction signal was evaluated in solutions of different pH relevant for the drug release. Fig. 3 A & B, (b) display the stability of the response for a 0.2 μM doxorubicin solution of pH 7.4 (A) and 5.2 (B) during a series of 12 successive measurements over a 60 min period of continuous operation. A highly stable response is observed in both solutions, with no apparent loss in sensitivity over this period. Such stability is indicated from the low relative standard deviations of 2.1% (A) and 4.8% (B), and reflects the efficiency of the *in situ* electrochemical cleaning step (that removes adsorbed drug from the surface). It should be pointed out that the pH 5.2 solution led to higher peak intensities and a slight peak shift (data not shown).

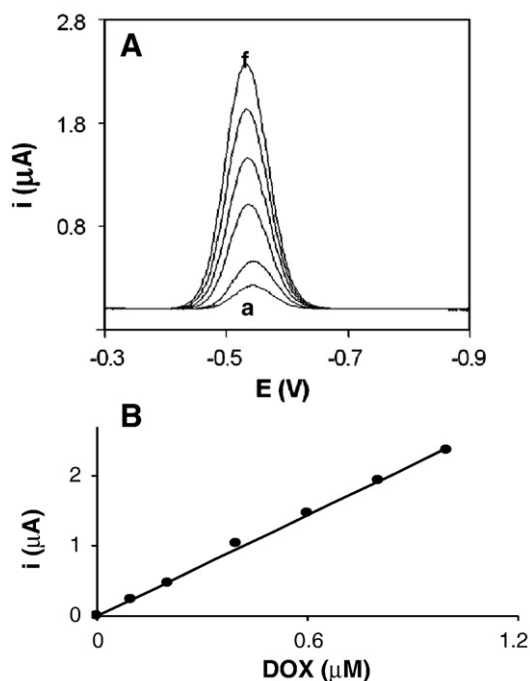


Fig. 2. (A) Square-wave voltammograms of doxorubicin at the concentrations of a–f: 0.1, 0.2, 0.4, 0.6, 0.8 and 1 μM . (B) The corresponding calibration plot. The experimental conditions include: 1 mL PBS buffer (pH = 7.4); scan from -0.3 V to -0.9 V using an amplitude of 25 mV, step of 2 mV and frequency of 25 Hz; equilibration time 20 s; nitrogen bubbling during the experiment; cleaning at -1.0 V for 40 s; and stirring during cleaning step.

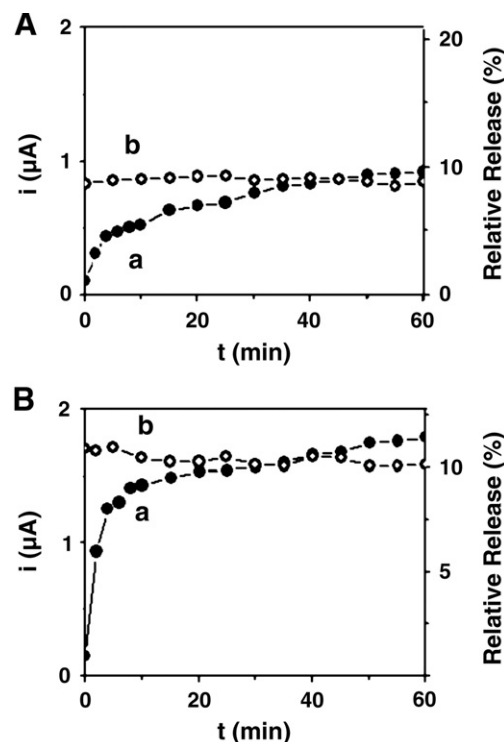


Fig. 3. (a) Current signals of the released doxorubicin at pH 7.4 (A) and 5.2 (B) over a 60-min period. (b) Current stability of the response for a 0.2 μM free doxorubicin solution at pH 7.4 (A) and 5.2 (B), respectively.

3.2. Voltammetric monitoring of doxorubicin release from liposomes in PBS buffer

Liposomes are spherical lipid vesicles with a bilayered membrane structure consisting of natural or synthetic lipid molecules. Liposomes have been widely used as pharmaceutical carriers in the past decade, accounting for ~50% of all the Food and Drug Administration (FDA) approved NP drug carrier formulations [4,22]. DOXIL, doxorubicin-loaded liposomes, was the first such product to be approved by the FDA for the treatment of AIDS associated with Kaposi's sarcoma and today is used in the treatment of a variety of cancers including ovarian, bladder, and breast cancers [23]. In this study, we chose clinically approved DOXIL as a model drug delivery NP system for the study of doxorubicin release kinetics using the new voltammetric monitoring system. It is important to understand the drug release kinetics of such NP systems in order to estimate effective dosing and to minimize systemic side effects. We chose DOXIL as a model drug delivery NP system for the study of release kinetics using the new voltammetric monitoring system.

In the *in vivo* environment, NPs will experience two general pH conditions: pH = 7.4 while circulating in the blood, and pH \approx 5 after being internalized by the cell (whether cancerous or normal) [2,22]. For this reason, we selected to monitor in real time the drug release kinetics in buffers at these two pH values (7.4 and 5.2). In the study, doxorubicin-loaded liposomes were prepared by taking commercially available DOXIL and removing all possible free doxorubicin through a washing step using an Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA) with a molecular weight cut-off of 10,000 Da.

The doxorubicin-loaded liposomes (DOXIL) were added to a PBS buffer at either pH = 7.4 or pH = 5.2. The free doxorubicin released from the liposomes was then measured with the voltammetric sensor using the protocol described in the experimental section. Fig. 3 A & B, (a) show the drug release kinetics in pH = 7.4 buffer and pH = 5.2 buffer, respectively. The release curves were obtained by monitoring the voltammetric signals of doxorubicin in 5 minute intervals over the

60 minute period immediately following the addition of the liposomes. The relative drug release (%) was calculated by normalizing the amount of released doxorubicin to the total encapsulated doxorubicin which was measured after breaking the liposomes via sonication and vortexing for 2 days. The measured total doxorubicin loading was consistent with what has been provided by the manufacture of DOXIL. The data of Fig. 3 show that for the 60 min over which the release kinetics were examined, there clearly appear two phases of release – a fast initial release followed by a slower sustained release. Measurement of the fast initial release is easier and more reliable with the new real-time monitoring capabilities of our electrochemical sensor. Traditional methods of measuring drug release require considerable sample preparation time, which means that the initial fast release has likely already occurred by the time monitoring or measurement begins. The ability to monitor in real-time from the initial sample loading is a major advantage of the direct electrochemical monitoring protocol described here.

The difference between the release kinetics profiles in the two different pH buffers is also worth noting. In pH = 7.4 buffer, the liposomes released 10 wt.% of their cargo within the first 60 min while approximately 12 wt.% was released in the pH = 5.2 buffer. The rate of release during the initial fast-release period is also higher for the low pH buffer, with a very rapid release (of up to 9%) during the first 10 min followed by a slow quasi-steady state release. In contrast, the release curve in neutral buffer is much smoother, with a rapid release (to ca. 4%) within the initial 5 min, followed by a gradual release sustained over the remaining 55 min. It takes around 10 min for 5 wt.% doxorubicin to be released in the neutral buffer compared to less than 5 min in the low pH buffer. One hypothesis is that at lower pH the lipid membrane is less stable which allows for a faster initial release. After the initial release, the drug release kinetics appears to be dominated by the diffusion of doxorubicin from the solid crystalline doxorubicin cluster encapsulated inside the liposomes. Note also (from Fig. S1,b) the intermediate kinetic profile observed in a pH = 6.4 buffer.

3.3. Voltammetric monitoring of doxorubicin release from liposomes in human serum

Besides measuring drug release kinetics from drug-loaded NPs, the new voltammetric monitoring system can detect drug concentration in blood in a user-friendly and accurate way. In clinical practice, cancer patients are treated with a bolus dosage of drugs solely based on their size and weight. However, determining the most effective dosing based solely on these criteria is not reliable due to differences in patient metabolism, tumor size, and reactions to drugs. Therefore, it would be very desirable to be able to monitor the drug concentration in the blood after administration in order to adjust future doses and maximize the therapeutic index. To this end we used our device to measure drug release kinetics of DOXIL in human serum. We chose to dilute the serum to 10% in PBS buffer in order to reduce non-specific binding and buildup of serum proteins onto the electrode surface. In clinical practice, a blood or serum sample from patients could be easily diluted in order to achieve the same results as presented here. The sensitivity of the device is sufficient such that a 10% dilution will not hinder the measurement of the drug (Fig. S2).

A new calibration curve was created for doxorubicin in the 10% serum medium (Fig. S3). The calibration curve shows good linearity in 10% human serum for both pH 7.4 and pH 5.2 (data not shown). We then measured the continuous release of doxorubicin from liposomes in 10% serum solution (pH = 7.4). As shown in Fig. 4, the doxorubicin release is much slower in 10% serum than in the PBS buffer. During the first 60 min, about 1.5 wt.% of the drug is released from the liposomes compared with about 10 wt.% in PBS buffer. There are several possible reasons for the slower release kinetics in serum. One is that serum proteins bind to the surface of the liposomes which impede the release of the doxorubicin. Another is that serum proteins bind to the

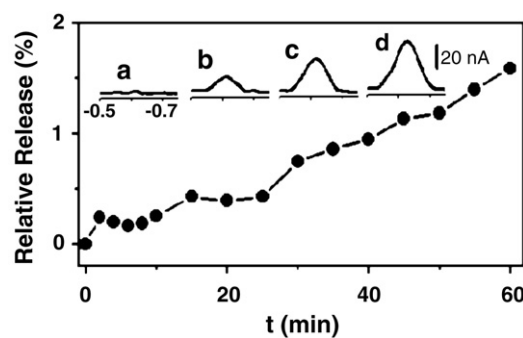


Fig. 4. Doxorubicin release kinetics from the liposomes in 10% serum PBS buffer (1X, pH = 7.4) over a 60-min period. Inset: typical square-wave voltammograms at (a) 0 min, (b) 20 min, (c) 40 min and (d) 60 min, respectively.

doxorubicin molecules after they are released from the liposomes, preventing an accurate voltammetric reading by the sensor. Still another reason could be that the decreased external osmotic pressure caused by the serum proteins shrinks the liposomes by drawing out water and therefore increasing the concentration of doxorubicin inside. The increased doxorubicin concentration promotes doxorubicin precipitation thus reducing the amount of free doxorubicin capable of diffusing across the membrane. The increased concentration of solutes (plasma proteins) outside of the liposomes also decreases the osmotic pressure driving the solute (doxorubicin) inside the liposomes across the lipid membrane. Further studies and characterizations are necessary to determine to what degree these factors affect the release kinetics of doxorubicin in serum.

3.4. General comments

Many synthetic and natural compounds have been proposed for cancer therapy and show great promise from *in vitro* studies, primarily due to their extremely high cytotoxicity [24]. The high toxicity of these compounds makes them effective anti-tumor drugs, but requires minimizing their exposure to healthy tissues. Recent advances in nanotechnology and drug delivery NPs successfully address this problem. Highly toxic compounds can be loaded inside NP carriers, shielding them from uptake and metabolism by healthy tissues. Upon arrival at the tumor site, their cargo can be released and taken up by the cancerous cells. There are many products currently in the pipeline that rely on this approach. However, it is unlikely that such drug delivery NPs will gain FDA approval for clinical use without detailed characterization of their drug loading yields and drug release kinetics. For many of these compounds, the traditional analytical tools cannot be used, particularly those drugs that lack a unique UV absorbance signature. Electrochemical monitoring offers an attractive solution to characterizing the drug release kinetics and loading yields of these drug delivery systems. Ongoing research in our laboratories aims at providing real-time monitoring capabilities for many novel compounds, such as marine-life derived drugs and hydrophobic drugs with limited water solubility. Progress will be reported shortly and separately.

4. Conclusions

We have demonstrated the use of voltammetric measurements for real-time monitoring of drug release kinetics from therapeutic NPs. By providing an analytical measurement in a timely, simple and cost effective fashion, the new voltammetric protocol offers direct and reliable assessment of the drug release kinetics from NP drug carriers. Unlike a recent potentiometric probe [14], the new voltammetric method has a broader scope, higher sensitivity and speed. This protocol also provides accurate detection of therapeutic molecules in serum. While the concept has been illustrated for the release of

doxorubicin from liposomes, it could be readily extended to monitor the release kinetics of other electroactive drugs from a wide range of different host therapeutic NPs. While many cancer drugs are electroactive and suitable for such voltammetric monitoring, the method is not suitable for study on the release of non-electroactive drugs. The specific surface activation conditions between successive runs would depend upon the specific drug, particle host and sample matrix. The new real-time monitoring capability thus offers considerable promise for characterizing the release properties of new drug delivery NPs.

Acknowledgements

LM acknowledges a fellowship from Spanish Science and Innovation Ministry (AP2005-0266). JW acknowledges a financial support from NIH (RO1 EB002189). LZ acknowledges a financial support from the University of California – San Diego (faculty start-up fund) and the Hellman Faculty Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.08.002.

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