Supporting Information

Distribution of diffusion times determined by fluorescence (lifetime) correlation spectroscopy

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S1. Details of changes in the program package FCS-CONTIN

S1.1. Modified subroutine USERK in FCS-CONTIN

```
FUNCTION USERK (JT,T,JG,G)
DOUBLE PRECISION PRECIS, RANGE
LOGICAL DOCHOS, DOMOM, DOUSIN, DOUSNQ, LAST, NEWPG1,
1 NONNEG, ONLY1, PRWT, PRY, SIMULA, LUSER
DIMENSION T(JT), G(*)
DIMENSION IHOLER(6)
COMMON /DBLOCK/ PRECIS, RANGE
COMMON /SBLOCK/ DFMIN, SRMIN,
1 ALPST(2), EXMAX, GMNMX(2), PLEVEL(2,2), RSVMNX(2,2), RUSER(551),
2 SRANGE
COMMON /IBLOCK/ IGRID, IQUAD, IUNIT, IWT, LINEPG,
1 MIOERR, MPKMOM, MQPITR, NEQ, NERFIT, NG, NINTT, NLINF, NORDER,
2 IAPACK(6), ICRIT(2), IFORMT(70), IFORMW(70), IFORMY(70),
3 IPLFIT(2), IPLRES(2), IPRINT(2), ITITLE(80), IUSER(50),
4 IUSROU(2), LSIGN(4,4), MOMNMX(2), NENDZ(2), NFLAT(4,2), NGL,
5 NGLP1, NIN, NINEQ, NNSGN(2), NOUT, NQPROG(2), NSGN(4), NY
COMMON /LBLOCK/ DOCHOS, DOMOM, DOUSIN, DOUSNQ, LAST,
1 NEWPG1, NONNEG, ONLY1, PRWT, PRY, SIMULA,
2 LUSER(30)
DATA IHOLER/1HU, 1HS, 1HE, 1HR, 1HK, 1H /
IF (JT.GT.NY .OR. JG.GT.NG+1 .OR. MIN0(JT,JG).LE.0) CALL
1 ERRMES (1,.TRUE.,IHOLER,NOUT)
USERK=((1+T(JT)/G(JG))**(-1.0))*((1+(RUSER(11)**2)*T(JT)/G(JG))**(-0.5))
RETURN
  END
```

S1.2 Sample of input data file to FCS-CONTIN

```
DATAFILE 01__BSAAlexa488_OD=1_120s_1
LAST -1.
GMNMX 1 2.7501E-04
GMNMX 2 3.1131E+01
IFORMT
(1e13.6)
```

```
IFORMY
(1E13.6)
IFORMW
(1E13.6)
NINTT -1.
DOUSNQ 0.10000E+01
IWT 2.
IQUAD 0.10000E+01
NERFIT 0.
NLINF 1.
IUSER 10 4.
RUSER 10 0.
LUSER 3 -1.
IUSROU 1 0.
IUSROU 2 0.
IPLRES 1 3.
IPLRES 2 3.
IPLFIT 1 3.
IPLFIT 2 3.
NQPROG 2 30.
NQPROG 1 30.
RUSER 11 0.1986
NG 0 100.
END
NY 140
```

Followed by 140 lines with values of delay time, then followed by 140 lines with values of correlation function, and, if available, followed by 140 lines with values of experimental weights; in the latter case IWT above has to be changed from 2. to 4.

NY is the number of points of the correlation function in the example above where it is set to 140 but can be any number up to 8192. Similarly NG is the number of grid points that can be selected, the upper limit is 500. NG = 100 is sufficient for the majority of applications. It should be noted that the computation time increases approximately as NG^3. Other lines in the sample of input data file do not need to be changed.

S2. Experimental part

S2.1 Materials

Atto 488 *N*-hydroxysuccinimidyl (NHS) ester, Nile red fluorescent dye, azobisisobutyronitrile (AIBN), ethylene glycol, PEG-600, poly(ethyleneimine) solution (50 wt.% in water), potassium dihydrogen phosphate, sodium phosphate dibasic dihydrate and Sephadex® G-25 were purchased from Sigma Aldrich Ltd. (Prague, Czech Republic). *N*-(2-Aminoethyl) methacrylamide hydrochloride (AEMA) was purchased from Polysciences

Europe GmbH (Mannheim, Germany). Alexa Fluor™ 405 NHS ester and 2,4,6-trinitrobenzene 1-sulfonic acid (TNBSA) solution were purchased from Thermo Fisher Scientific (Czech Republic). Poly(DL-lactide)-*b*-poly(ethylene oxide) (PLA_{273}-b-PEO_{113}) was bought from Polymer Source (Canada). *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was prepared according to ref.[1]

S2.2 Synthesis and preparation

Polymer synthesis

Polymer L119 (PHPMA-*co*-AEMA).

The HPMA (500 mg, 3.50 mmol), AEMA (50 mg, 0.30 mmol) and AIBN (50 mg, 0.30 mmol) were dissolved in ethylene glycol (2 mL), and the reaction mixture was left unstirred overnight at 70 °C under an argon atmosphere. Water (5 mL) was added to the reaction mixture, and it was dialyzed (molecular weight cut-off 3500 Da) against water for 72 h and freeze-dried to give the desired product marked as L114 (315.1 mg). The content of the $–NH_2$ groups was determined using a TNBSA assay according to ref.[2] (0.29 mmol/g of $–NH_2$ groups).

L114 (100 mg, 0.029 mmol of $–NH_2$ groups), $Na_2HPO_4 \cdot 2\ H_2O$ (0.383 g, 1.98 mmol) and KH_2PO_4 (45 mg, 0.33 mmol) were dissolved in water (2 mL), and the solution was cooled to 0 °C. Atto 488 NHS ester was added (0.3 mg), and the mixture was stirred overnight. The crude product was purified on a Sephadex® G-25 column using water as the mobile phase and freeze-dried to give product L119 (85 mg). The content of Atto 488 was determined spectrophotometrically to be 0.06 wt. % ($\lambda_{absorption}$ = 501 nm and ε = 90 000 L/mol·cm).

Polymer L118 was prepared using the same procedure like L119, except that PEG-600 was used for dissolution of the reaction mixture instead of ethylene glycol.

Nanoparticle preparation

PLA_{273}-*b*-PEO_{113} nanoparticles were prepared by microfluidics technique. The PLA_{273}-*b*-PEO_{113} was dissolved in acetonitrile in order to produce a polymer solution with a final concentration of 2.5 mg/mL^{-1}, to the prepared polymer solution was added 1 $\mu L/mL^{-1}$ of fluorescent dye Nile red in order to stain the final product. Nanoparticles were produced using the microfluidic device setup Dolomite (Dolomite Microfluidics, U.K) which was equipped with a glass Micromixer chip with 12 mixing stages microchanels of 50μm x 125μm (depth x width). The polymer solution was pumped through the middle channel and ultrapure water flowed through the side channels using two independent Dolomite Mitos P-pumps (U.K.) controlled via PC software. The tested flow rates range between 20 to 400 $\mu L/mL^{-1}$ and 500 to 1000 $\mu L/mL^{-1}$ in the organic and water channels respectively, resulting in flow ratios from 0.04 to 0.8. The resulted nanoparticles were collected in vials and after solvent evaporation characterized by dynamic light scattering using a Malvern Nano ZS90 (Malvern, UK).

S2. 3 Methods

Size exclusion chromatography

The molecular weight was determined using size exclusion chromatography (SEC); the system contained a Deltachrom SDS030 pump (Watrex Co., Prague, Czech Republic), a MIDAS autosampler (Spark HOLLAND B.V., Netherlands) an Optilab t-rEX refractive index detector and DAWN Heleos II multiangle light-scattering detectors (MALSs) (both from Wyatt Technology Corporation, USA). L119 was measured using TSK gel G3000SWxl column (Agilent, Prague, Czech Republic) in the mixture of methanol and acetate buffer (80/20) as the mobile phase. L121 was measured using CATSEC 1000 column (Agilent, Prague, Czech Republic) and 0.1 M sodium nitrate/0.1 wt. % trifluoroacetic acid in water as the mobile phase.

Dynamic light scattering

Dynamic light scattering (DLS) were performed using an ALV CGE laser goniometer (ALV-Laser Vertriebsgesellschaft m-b.H., Langen, Germany), which consists of a 22 mW HeNe linear polarized laser operating at a wavelength of 632.8 nm, an ALV 6010 correlator, and a pair of avalanche photodiodes operating in the pseudo cross-correlation mode. The copolymer solutions were placed in 10 mm diameter glass cells and maintained at a constant temperature of 20 ± 1 °C in all experiments. Data were collected using ALV Correlator Control software V.3.0.3.8. 05/2008 and the counting time varied for each sample from 300 to 600 s. The recorded data were exported and further analyzed by CONTIN analytical software providing regularized fit or by Pearson V analytical fit[3] of distribution functions of hydrodynamic radii, $A(R_h)$.

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) measurements were performed on an IX83 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a FLIM/FLCS upgrade kit (PicoQuant GmbH, Berlin, Germany). The samples were excited by an LDH-D-C-485 laser diode emitting 482 nm light, driven by a PDL 828 Sepia II driver in picosecond pulsed mode at a 20 MHz repetition rate (both devices: PicoQuant) through the 488 nm dichroic mirror built into the IX83 scan head. An Olympus UPlanSApo water immersion objective (60x, 1.2 NA) delivered the excitation light into a diffraction-limited spot and collected the emitted fluorescence. The fluorescence signal passed through a Semrock 600/50 or 520/35 nm BrightLine emission filters and was detected by a hybrid photomultiplier (PMA Hybride-40 from PicoQuant) operated in photon counting mode. Photon counts were recorded using a PicoHarp300 TCSPC module in a T3 time tagging mode.[4] The measurements were performed at 23 ± 1 °C over a period of 10 or 20 min. The SymPhoTime64, ver. 2.1 software

(PicoQuant) was used for data acquisition and FCS autocorrelation function calculation. The autocorrelation functions were exported to text files and further processed by FCS-CONTIN software.

References

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[2] Cayot, P.; Tainturier, G. The Quantification of Protein Amino Groups by the Trinitrobenzenesulfonic Acid Method: A Reexamination. *Anal. Biochem.* **1997**, *249*, 184-200.

[3] Jakeš, J. Regularized Positive Exponential Sum (REPES) Program - A Way of Inverting Laplace Transform Data Obtained by Dynamic Light Scattering. *Collection Czechoslovak Chem. Commun.* **1995**, *60*, 1781-1797.

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