Supplementary Material (ESI) for Molecular BioSystems

Supporting Information

Multifunctional Polymeric Micelles with Folate-Mediated Cancer Cell Targeting and pH-Triggered Drug Releasing Properties for Active Intracellular Drug Delivery

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Contents

1. Quantitative introduction of hydrazide groups to the PBLA block

As described in the main text, ester-amide exchange (EAE) aminolysis reaction was adopted in this study as one of the most convenient synthetic methods to simply and efficiently introduce hydrazide groups to the side chain of poly(ethylene glycol)-poly(β-benzyl L-aspartate) [PEG-PBLA] block copolymers, and thereby, it facilitates the preparation of folate-poly(ethylene glycol)-poly(aspartate hydrazone adriamycin) [Fol-PEG-p(Asp-Hyd-ADR)] block copolymers with desired contents of hydrazide group drug-binding linkers. This section explains the detailed supporting information on the quantitative introduction of hydrazide groups to the PBLA block, by tracking EAE reaction in an NMR tube.

1.1. Experimental procedures

Materials and device

Hydrazine (H_2NNH_2, anhydrous, MW=32.05) was purchased from Tokyo Kasei Organic Chemicals Co., Ltd., Japan, and dried over calcium hydride followed by the distillation.

N,N-dimethylformamide (DMF), N,N-dimethylformamide-d_7 (DMF-d_7), dimethylsulfoxide (DMSO), dimethylsulfoxide-d_6 (DMSO-d_6), Acetic anhydride (AA) and diethyl ether were purchased from Wako Pure Chemical Industries, Co., Ltd., Japan. DMF was distilled twice following standard procedures.

Tracking EAE reaction in an NMR tube

Amphiphilic block copolymer poly(ethylene glycol)-poly(β-benzyl L-aspartate) (PEG-PBLA) was synthesized and characterized as described in the main text. Obtained PEG-PBLA (Mw=18,112, Mw/Mn=1.09) was freeze-dried from benzene prior to ester-amide exchange (EAE) reaction. In order to track the reaction proceeding in real time and to provide quantitative information about specific reaction species, EAE reaction between PEG-PBLA and hydrazine was carried out in an NMR tube. PEG-PBLA (50 mg) was freeze-dried from benzene in a flask and dissolved in 1 ml of DMF-d_7. After 1.49 μg of hydrazine (0.5-fold with respect to benzyl groups) was added using a micro syringe, the mixed solution was transferred from the flask to an NMR tube in the presence of Ar, followed by sealing the tube under liquid N_2 environment while vacuuming. Subsequently, NMR was taken every 4 minute interval. In order to assign the peaks appearing, solvents and materials such as benzyl alcohol, hydrazine, DMF-d_7, that were expected to produce during the reaction were investigated by ^{1}H-NMR prior to the reaction (on the left below panel of figure S1).

1.2. Results and discussion

In this study, it is suggested that PEG-p(Asp-Hyd) can be successfully prepared by a one-step synthetic method using EAE reaction which is effective to introducing amine groups to the side chain of PEG-PBLA directly and quantitatively (Figure S1). In particular, this method is also considered of great convenience when designing and synthesizing novel block copolymers conjugated with biologically active materials that are unstable under acidic conditions.

Figure S1 shows EAE reaction proceeds in an NMR tube. On the right panel of Figure S1, we can see that the peaks from hydrazine shifted gradually to the position of hydrazide conjugated to the side chain of PEG-PBLA via an amide bond, while the peaks from benzyl alcohol appeared demonstrating substitution with hydrazine proceeds. The reaction was revealed to be over within 1 h. In particular, it is notable that EAE reaction did not induce any side-reaction inducing interlinking or crosslinking. ^{1}H-NMR peak of the products showed that as many as added hydrazine molecules were introduced to the side chain of PEG-PBLA and unreacted benzyl esters remained stable. For the calculation of precise substitution ratio, hydrazide groups, that usually show broadening peaks, were labeled with acetyl groups as shown in Figure S2. The results also clearly indicated that EAE reaction is quantitative and introduced hydrazide groups are active.

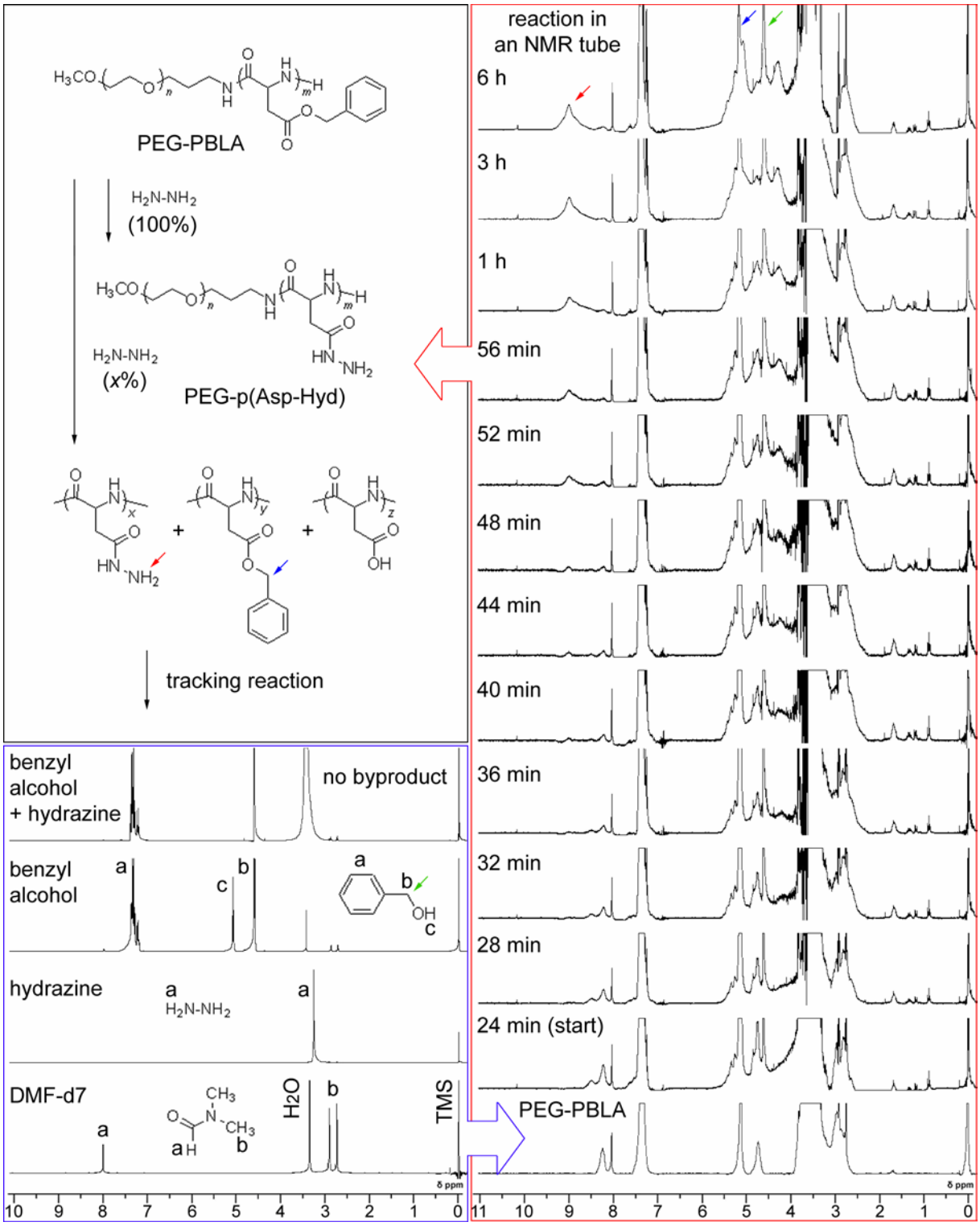

Figure S1. Synthesis of PEG-p(Asp-Hyd) via ester-amide exchange (EAE) aminolysis and tracking the reaction in an NMR tube. (DMF-d_7 at 40°C).

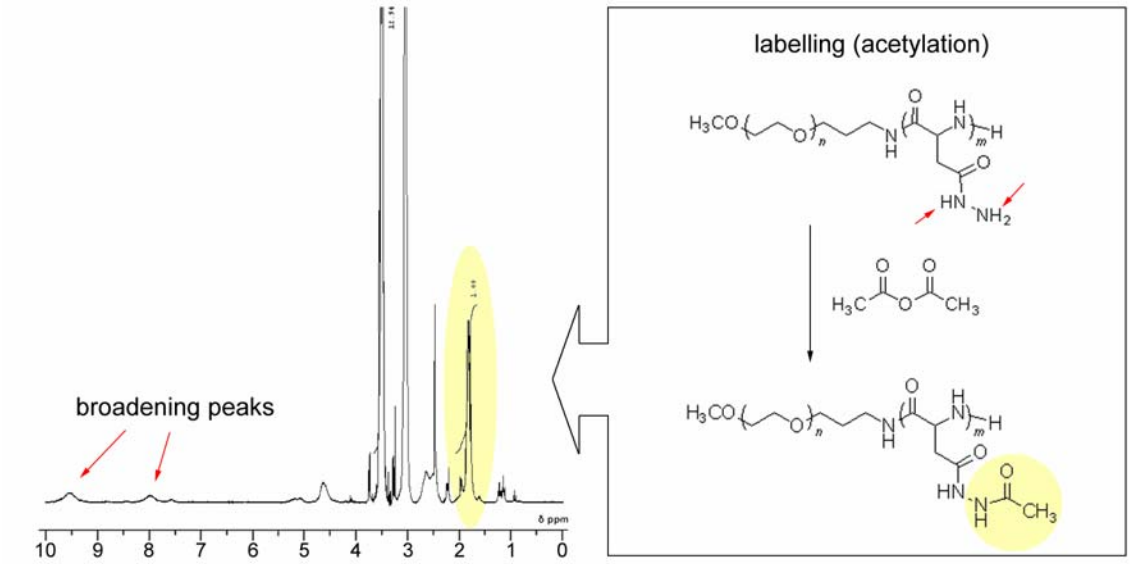

Figure S2. Precise quantification by labeling hydrazide groups with acetyl groups.

1.3. Conclusion

This supporting information has elucidated a new synthetic method to introduce hydrazide groups, as drug-binding moieties, to PEG-PBLA quantitatively, which is described in the main text. As shown, the EAE reaction provides a simple and convenient methodology to prepare intracellular pH-sensitive polymeric micelles with various drug-loading contents.

2. Correlation between number of linkers and drug release profile of the micelle

It is generally considered drug-loading contents play a crucial role to prepare stable micelle formation. Nevertheless, optimum compositions for this system still remain to be determined from the aspects of drug release speed as well as the maintenance of stability. For these reasons, PEG-p(Asp-Hyd-ADR) block copolymers with varying drug-loading contents were prepared by changing the number of hydrazide drug-binding linkers via a new synthetic method, ester-amide exchange (EAE) aminolysis reaction. In this supporting information section, pH-sensitive drug release behaviors of the micelle employing various contents of hydrazides and carboxylates are shown. By this effort, the optimum compositions and the way of controlling drug release profile of the micelle in the main text become elucidated.

2.1. Preparation of the micelles with various drug-loading contents

By the synthetic method described in the supporting information section 1, PEG-p(Asp-Hyd) block copolymers with various contents of hydrazides were prepared, and conjugated with ADR to

give drug-bound poly(ethylene glycol)-poly(aspartate hydrazide-adriamycin) [PEG-p(Asp-Hyd-ADR)] block copolymers as reported elsewhere (Y. S. Bae, S. Fukushima, A. Harada, K. Kataoka, Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: Polymeric micelles that are responsive to intracellular pH change, Angew. Chem., Int. Ed., 42(38), 4640-4643 (2003)). The PEG-p(Asp-Hyd- ADR) block copolymers were dissolved in DMAc (10 ml) to prepare the micelles by a dialysis method. Physicochemical properties and pH-sensitive drug release behaviors of prepared micelles were evaluated by dynamic light scattering (DLS) and reversed-phase liquid chromatography (RPLC), respectively.

2.2. Results and discussion

Characterization of prepared micelles

Conjugation between PEG-p(Asp-Hyd) and ADR was successfully carried out, and drug-binding ratio was 90.7% with respect to each hydrazide group. The reason why some hydrazide groups, approximately 10%, remained unreacted is probably due to the steric hindrance between ADR molecules. Nevertheless, reaction ratio was extremely high with satisfactory drug-loading efficiency, which shows most of hydrazide groups remained active after introduction. Interestingly, the physicochemical properties of these micelles, such as size and drug release profile, changed depending on the contents of hydrazide groups for drug binding, in other words, drug-loading contents. Table S1 shows substitution ratio of the hydrazides in each block copolymer and size of prepared micelles, respectively. It is clearly demonstrated that the size of the micelle increased as substitution ratio increased, and it reached approximately 50 nm, which was monitored by using DLS. In particular, only the micelles with substitution ratio over 53% maintained structural stability even after filtration with a 0.22 μm pore size, indicating the optimum amounts of drug binding moieties required for the preparation of stable micelles.

Table S1. Change in size and stability of the micelles with varing number of drug linkers

	Substitution ratio of hydrazide groups				
	3 (11 %)	7 (25 %)	15 (53.6 %)	22 (79 %)	28 (100 %)
Micelle size	10.9 nm	12.4 nm	36.9 nm	43.7 nm	52.8 nm
Sterilization using filters	×	×	○, 33.1 nm	○, 40.7 nm	○, 48.1 nm
(polydispersity index, μ/Γ^2) [a]	ND [b]	ND [b]	(0.2019)	(0.1790)	(0.1698)
pH-Sensitivity [c]	□	□	○	○	●

[a]The micelles were filtered using 0.22 μm filter units (Millex-HV, Millipore, Co., Ltd., USA).

[b]ND denotes not detected.

[c]pH-Sensitive drug release profile was very rapid (□), moderate (○) and extremely slow (●).

In the meantime, RPLC analysis provided very intriguing information on the relationship between substitution ratio of hydrazide groups and drug release profile of the micelles (Figure S3). Drug release speed became slower as substitution ratio of hydrazide groups. However, interestingly, the micelles from PEG-p(Asp-Hyd) with 100% substitution ratio were unlikely to release drugs over 48 h. On the other hand, the micelle from PEG-p(Asp-Hyd) with substitition ratio of 25% or less released drugs within 1 h.

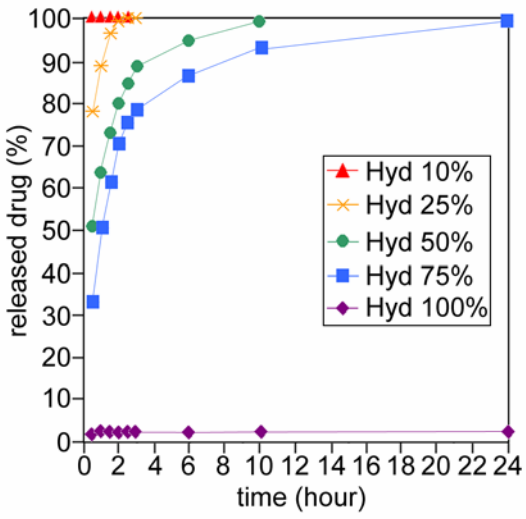

Figure S3. Correlation between substitution rate of drug-binding hydrazide linkers and drug release profile of the micelles.

This critical change in drug release may be due to stability of the micelle core. Stable micelle cores may prevent the diffusion of hydronium ions as well as hydrophobic drugs, and therefore, drug-binding linkers cannot be easily cleaved. Or even when they were cleaved, drugs cannot be diffused out from the micelle. In this regard, carboxylate groups remained in the block copolymers may play an important role to control drug release by modulating chemical conditions in the micelle core. Indeed, carboxylate groups become deprotonated and hydrophobic under acidic conditions. For these reasons, it is expected that carboxylate groups give structural heterogeneity in the micelle core to relieve steric hindrance between ADR molecules. Nothing is clear how they exactly affect the release mechanism at present, the results show that substitution ratio of hydrazide, namely the balance between hydrazide and carboxylate groups in the micelle core, induces the changes in drug release speed as well as drug-loading contents. Consequently, it is strongly suggested that the counter charge and steric hindrance in the micelle core should be carefully determined considering stability and drug release property of the micelle.

2.3. Conclusion

In this supporting information section, intracellular pH-sensitive polymeric micelles with various drug-loading contents were prepared and characterized focusing on their drug release profile. Indeed, it has been revealed that the micelles from PEG-p(Asp-Hyd-ADR) with higher substitution ratio of hydrazide groups gives smaller diameters, and drug release from the micelles becomes slower. These interesting experimental results demonstrate that at least 53% of ADR loading is inevitable to prepare stable micelles. Even though further studies might be required to elucidate relationship between the structure of the micelle core and the drug release profile, it is concluded that the optimum compositions of PEG-p(Asp-Hyd-ADR) block copolymers produce the best performance of the pH-sensitive micelles, while this fact differentiates the micelles from the typical prodrugs with acid-cleavable linkers. On the basis of these results, compositions of block copolymers to prepare FMA and MA were determined.