Modulation of zeta potential and molecular mobility on polyrotaxane surfaces promotes osteoblastic and adipogenic differentiation of mesenchymal stem cells

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Abstract

Statement of Purpose: Polyrotaxanes are supermolecules composed of cyclic molecules (e.g. a-cyclodextrins (a-CDs)) threaded onto linear polymer chains (e.g. poly (ethylene glycol) (PEG)). The a-CDs have an ability to move along the PEG chains, which referred to as molecular mobility. Previously, we designed polyrotaxane-based cell adhesive surfaces and succeeded in modulating cellular functions by controlling the molecular mobility of the polyrotaxane surfaces.[1] For instance, polyrotaxane surfaces with low mobility tend to promote osteoblast differentiation of mesenchymal stem cells (MSCs), whereas surfaces with high mobility tend to enhance adipocyte differentiation. Although we clarified the contribution of the mobility of polyrotaxanes to the guidance of cell functions, little is known about how the variety and properties of functional groups on polyrotaxane surfaces influence cellular functions. In this study, we designed mobile polyrotaxane surfaces with functional groups that differ in their electronic charge and hydrophobicity.[2] The contact angle and zeta potential were measured to evaluate the physicochemical properties of the surfaces. Osteoblast and adipocyte differentiation was induced in hMSCs, and differentiation was evaluated by Alizarin red S staining and Oil red O staining, respectively.

Methods: Polyrotaxanes with the CH_3 group as a hydrophobic and nonionic group (CH_3-PRX), OH group as a hydrophilic and nonionic group (OH-PRX), NH_2 group as a cationic group (NH_2-PRX), and sulfo group (SO_3H-) as an anionic group (SO_3H-PRX) were coated on tissue culture polystyrene (TCPS). The static contact angles of the polyrotaxane surfaces were measured using both the sessile-drop method in air and the captive bubble method in water with an optical contact angle meter. The zeta potential of the polyrotaxane surfaces was measured using an electrophoretic light scattering spectrometer with a quartz flow cell. To induce osteoblast and adipocyte differentiation, hMSCs were cultured using an hMSC Growth Medium Bullet Kit at 37° C and then the growth medium was replaced with an osteogenic or adipogenic differentiation medium. The differentiation medium was replaced with fresh medium every 3 to 4 d. The adherent cells were observed under a microscope. Fourteen days after osteoblast differentiation was induced, the cells were stained with Alizarin red S to evaluate hMSC mineralization. Fifteen days after adipocyte differentiation was induced, the cells were stained with Oil red O to evaluate the accumulation of hMSCs in intracellular lipid vesicles.

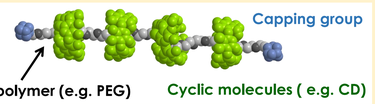
Results: When the contact angles of water on the polyrotaxane surfaces were measured using the sessile-drop method in air, the values of contact angles were within 80° –100° , except for the TCPS surface with an angle of 74° . When the static contact angles were measured using the captive bubble method in water, the values were as follows: CH_3-PRX (60.0±2.4°) > OH-PRX (52.9±4.0°) > NH_2-PRX (43.0±1.4°) > SO_3H-PRX (22.2±2.0°). The contact angle hysteresis calculated by the difference between the contact angles of water droplets and air bubbles was as follows: CH_3-PRX < OH-PRX < NH_2-PRX < SO_3H-PRX. We have previously reported that the contact angle hysteresis correlates with molecular mobility on polyrotaxane surfaces, and these results suggest that the molecular mobility of PRX surfaces changes depending on functional groups. The zeta potentials for the CH_3-PRX, OH-PRX, NH_2-PRX, and SO_3H-PRX surfaces were –24.6±2.8, –29.3±2.8, –16.3±6.3, –9.9±0.7, and –33.6±3.4, respectively. To evaluate the osteoblast differentiation of hMSCs cultured on each surface, calcium mineralization was detected using Alizarin red S after a 14-d induction of osteoblast differentiation. The stained area on SO_3H-PRX was the largest among those on all the surfaces, whereas only a small area was stained on NH_2-PRX. Interestingly, the area stained with Alizarin red S tended to increase with the negative charge on the surfaces. To evaluate the adipocyte differentiation of hMSCs cultured on each surface, lipid vesicle accumulation was detected using Oil red O after a 15-d differentiation induction. The stained areas on the OH-PRX, NH_2-PRX, and SO_3H-PRX surfaces were larger than those on the PRX and CH_3-PRX surfaces. This result suggests that the highly molecular mobility of polyrotaxane surfaces contributes to the promotion of adipocyte differentiation. Previous studies have suggested that hard materials and less mobile surfaces enhance osteogenesis, whereas soft materials and highly mobile surfaces enhance adipogenesis, indicating that the surfaces suitable for osteoblast differentiation are not the same as those for adipocyte differentiation. In contrast, SO_3H-PRX surfaces are suitable for inducing both osteoblast and adipocyte differentiation.

Conclusions: Two independent parameters, high molecular mobility and negative charge on polyrotaxane surfaces may not offset the effect to accelerate both differentiation. Therefore, the selection of functional groups plays a crucial role in cell manipulation using polyrotaxane-based biomaterials.

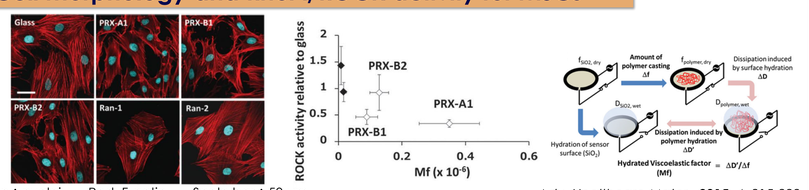
Introduction

Polyrotaxane (PRX)

PRX is a supermolecule with cyclic molecules (eg, cyclodextrins (CDs)) threaded onto an axle liner polymer (eg, poly(ethylene glycol) (PEG)).



Cell morphology and RhoA/ROCK activity for MSCs



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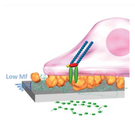
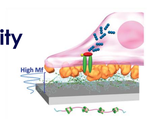
◆PRX-surfaces with Less molecular mobility

RhoA/ROCK activity ↑
Osteogenic differentiation



◆PRX-surfaces with highly molecular mobility

RhoA/ROCK activity ↓
Adipogenic differentiation



Experimental section

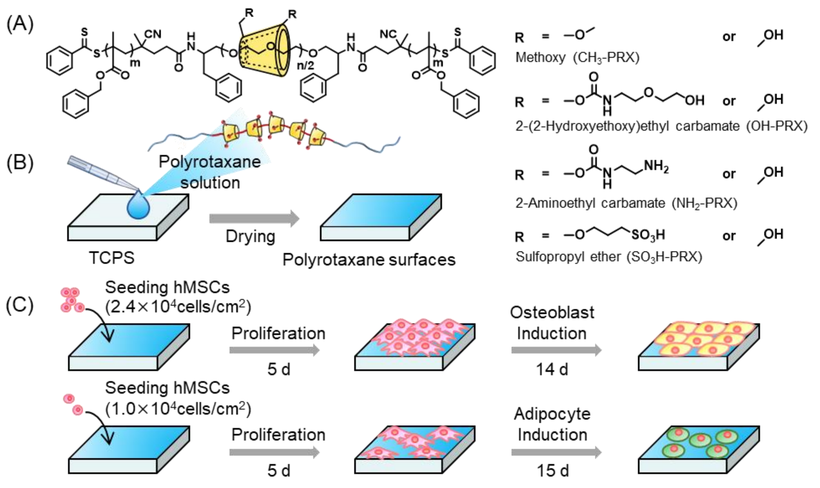


Figure 1. Chemical structure of polyrotaxane triblock polymers with functional groups (A). Fabrication of polyrotaxane surfaces with functional groups (B). Experimental procedure for hMSC differentiation on polyrotaxane surfaces (C).

Characterization

Table 1) Characterizations of polyrotaxane-based surfaces.

Code[a]	M_n of PEG[b]	M_n of PBzMA[b]	Number of a-CDs[b]	Number of functional groups[c]	Element (atom%)[d] C1s	O1s	N1s	S2p
PRX	20,000	115,200	89.8	0 (0)	95.8±0.3	4.0±0.5	0.2±0.1	0.0±0.0
CH_3-PRX	20,000	115,200	89.8	312 (3.5)	94.0±2.4	5.8±2.3	0.3±0.1	0.0±0.0
OH-PRX	20,000	115,200	89.8	392 (4.4)	77.7±0.4	20.4±0.4	1.9±0.0	0.0±0.0
NH_2-PRX	20,000	115,200	89.8	310 (3.5)	78.8±0.2	19.1±0.3	2.1±0.1	0.0±0.0
SO_3H-PRX	20,000	115,200	89.8	568 (6.3)	92.6±2.7	6.5±2.3	0.2±0.2	0.3±0.2

[a] As a base surface, tissue culture polystyrene (TCPS) was used. PRX, PEG, PBzMA and a-CD indicate polyrotaxane, poly(ethylene glycol), poly(benzyl methacrylate) and a-cyclodextrin, respectively. The PBzMA -*block*-(*X*-PRX)-*block*-PBzMA triblock copolymer-coated TCPS are coded as '*X*-PRX', where *X*- indicates the functional groups modified to PRX: methoxy (CH_3-), 2-(2-hydroxyethoxy)ethyl carbamate (OH-), 2-aminoethyl carbamate (NH_2-), sulfopropyl ether (SO_3H-).[b] Determined by ^{1}H NMR in DMSO-d_6 or D_2O containing NaOD. [c] Determined by ^{1}H NMR in DMSO-d_6. Values in parentheses indicate the number of each functional group per threading a-CDs.[d] Elemental composition of carbon, nitrogen, oxygen and sulfur determined by XPS. Expressed as the mean ± SD; *n* = 3.

Table 2) Physiochemical properties of polyrotaxane-based surfaces.

Code[a]	Contact angle measured by water droplets method (θ)[e]	Contact angle measured by air bubble method (θ)[e]	Contact angle hysteresis (θ)[g]	Zeta potential (mV)[h]
PRX	93.8±1.3	75.5±1.5	18.3±1.1	-24.6±2.8
CH_3-PRX	85.0±1.7	60.0±2.4	25.0±3.9	-29.3±2.8
OH-PRX	83.5±1.1	52.9±4.0	30.7±5.1	-16.3±6.3
NH_2-PRX	80.8±2.1	43.0±1.4	37.8±2.8	-9.9±0.7
SO_3H-PRX	86.5±2.7	22.2±2.0	64.3±3.8	-33.6±3.4

[a] As a base surface, tissue culture polystyrene (TCPS) was used. PRX, PEG, PBzMA and a-CD indicate polyrotaxane, poly(ethylene glycol), poly(benzyl methacrylate) and a-cyclodextrin, respectively. The PBzMA -*block*-(*X*-PRX)-*block*-PBzMA triblock copolymer-coated TCPS are coded as '*X*-PRX', where *X*- indicates the functional groups modified to PRX: methoxy (CH_3-), 2-(2-hydroxyethoxy)ethyl carbamate (OH-), 2-aminoethyl carbamate (NH_2-), sulfopropyl ether (SO_3H-).[e] Determined by contact angle measurement of water droplets in air. Expressed as the mean ± SD; *n* = 4. [f] Determined by contact angle measurement of air bubbles in water. Expressed as the mean ± SD; *n* = 4. [g] Measured by a difference between contact angle of water droplets in air and air bubbles under water. Expressed as the mean ± SD; *n* = 4. [h] Determined with an electrophoretic light scattering spectrophotometer. Expressed as the mean ± SD; *n* = 3.

Protein adsorption

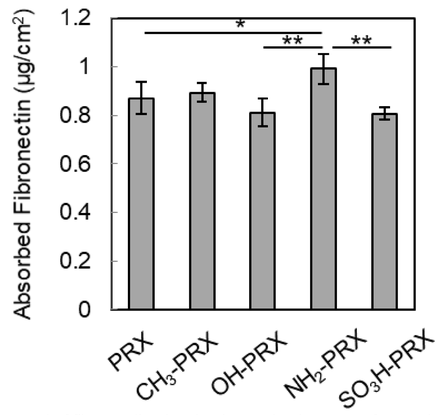


Figure 2. Amount of fibronectin absorbed on polyrotaxane surfaces, determined via a micro BCA assay. Polyrotaxane surfaces were incubated with human fibronectin in PBS solution for 3 h at 37° C and then washed with PBS solution to remove unabsorbed fibronectin. Absorbed fibronectin was extracted with 5% SDS and 0.1 N NaOH aqueous solution, and the amount extracted was determined using a micro BCA kit. All the measurements were obtained on four different surfaces for each polyrotaxane surface. Statistical analyses were conducted using one-way ANOVA and Tukey HSD method. ***p < 0.001, **p < 0.01, and *p < 0.05 indicate significance.

Cellular Proliferation

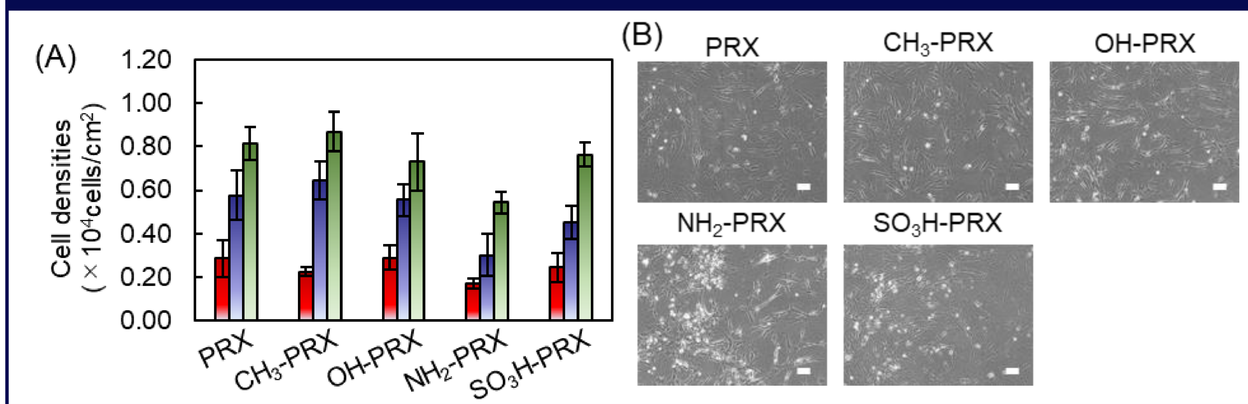


Figure 3. Proliferation of adherent hMSCs on PRX, CH_3-PRX, OH-PRX, NH_2-PRX, and SO_3H-PRX during the 3-d cultivation period. The hMSCs were seeded at a density of 6.0 × 10^3 cells/cm², and the cell density was calculated on days 1 (red), 2 (blue), and 3 (green). All the measurements were made on four different surfaces for each polyrotaxane surface (A). Phase-contrast microscopic images of hMSCs cultured on PRX, CH_3-PRX, OH-PRX, NH_2-PRX, and SO_3H-PRX surfaces after 1 d of culture. Scale bar: 100 μm (B).

Cellular differentiation

Adipogenic differentiation

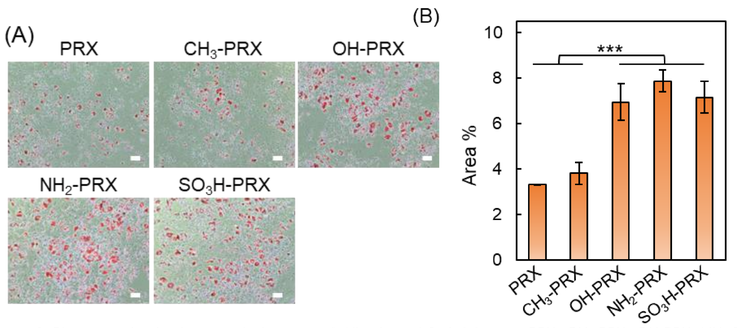


Figure 4. Phase-contrast microscopic images of Alizarin red S staining on PRX, CH_3-PRX, OH-PRX, NH_2-PRX, and SO_3H-PRX. The hMSCs were seeded at a density of 2.4 × 10^4 cells/cm² and expanded for 5 d; osteoblast differentiation was then induced. Fourteen days after differentiation induction, calcium was stained with Alizarin red S to evaluate mineralization. Scale bar: 200 μm (A). Percentage of stained area on each surface was calculated using Image J. All the measurements were obtained on three different surfaces for each polyrotaxane surface; averages were calculated from four different images on each surface. Statistical analyses were conducted using one-way ANOVA and Tukey HSD method. ***p < 0.001, **p < 0.01, and *p < 0.05 indicate significance. (B).

Osteoblast differentiation

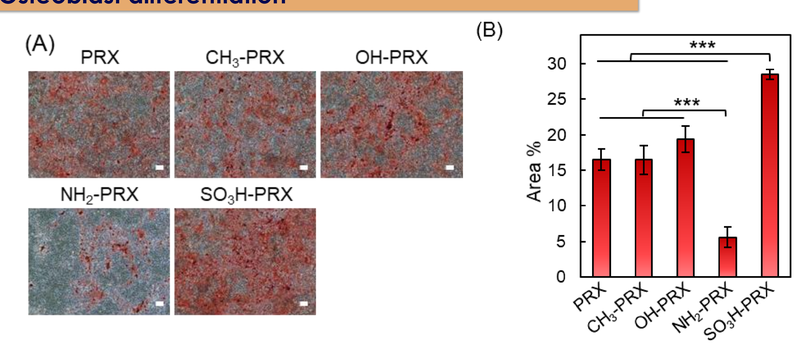


Figure 5. Phase-contrast microscopic images of Oil red O staining on PRX, CH_3-PRX, OH-PRX, NH_2-PRX, and SO_3H-PRX. The hMSCs were seeded at a density of 1.0 × 10^4 cells/cm² and expanded for 5 d; adipocyte differentiation was then induced. Fifteen days after differentiation induction, the lipid vesicle was stained with Oil red O to evaluate lipid vesicle accumulation. Scale bar: 100 μm (A). Percentage of stained area on each surface calculated using Image J. All the measurements were obtained on three different surfaces for each polyrotaxane surface; averages were calculated from three different images on each surface. Statistical analyses were conducted using one-way ANOVA and Tukey HSD method. ***p < 0.001, **p < 0.01, and *p < 0.05 indicate significance. (B).

Relationship between cellular differentiation and material properties of polyrotaxane surfaces

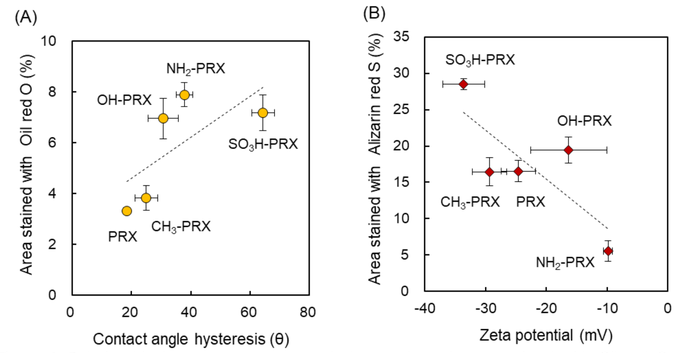


Figure 6. Relationship between contact angle hysteresis and area stained with Oil red O (A) and zeta potential and area stained with Alizarin Red S (B).

When polyrotaxane with the same number of threading a-CDs was used as a base polymer for modifying different functional groups, the zeta potential on the polyrotaxane surfaces may contribute to enhancing osteoblast differentiation. As the reason for enhancing osteoblast differentiation, the negative charge on SO_3H-PRX surfaces may promote an interaction with specific proteins. We previously reported that soluble sulfonated polyrotaxanes form electrostatic complexes with growth factors involved in cell differentiation and enhance the activity of growth factors. Furthermore, it has been clarified that sulfonated polyrotaxane surfaces also interact with various growth factors and drastically enhance cell functions. Although the detailed mechanism is unclear, SO_3H-PRX surfaces may act like glycosaminoglycans with a large number of sulfo groups, improving the maintenance of protein activity and cytocompatibility of polymeric materials. Although the detailed mechanism is not clear, we found that polyrotaxane mobility and surface zeta potential may not offset the effect to promote osteoblast and adipocyte differentiation.

Conclusions

Four types of polyrotaxane surfaces with different functional groups, namely, CH_3, OH, NH_2, and SO_3H, were fabricated using a drop cast method. Although the NH_2-PRX surface adsorbed a larger amount of fibronectin compared to other surfaces, cell adhesion and proliferation were limited on this surface. Interestingly, cells on the SO_3H-PRX surfaces underwent enhanced osteoblast and adipocyte differentiation. Presumably, two independent parameters, high molecular mobility and negative charge on SO_3H-PRX surfaces, may not offset the effect to accelerate both differentiation. Therefore, the selection of functional groups plays a crucial role in cell manipulation using polyrotaxane-based biomaterials. A combination of chemical modification and mobility tuning of polyrotaxane surfaces is expected to be a promising approach for constructing more appropriate culture environments according to cell type and differentiation lineage.

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