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An in-situ Dynamic Continuum of Supramolecular Phosphoglycopeptides Enables Formation of 3D Cell Spheroids

Huaimin Wang,[a] Junfeng Shi,[a] Zhaoqianqi Feng,[a] Rong Zhou,[a] Shiyu Wang,[b] Avital A. Rodal,[b] and Bing Xu*[a]

Abstract: Higher-order assemblies of proteins, with a structural and dynamic continuum, is a new paradigm in biology, but these insights have yet to be applied in designing biomaterials. Dynamic assemblies of supramolecular phosphoglycopeptides (sPGPs) transform a 2D cell sheet to 3D cell spheroids. A ligand-receptor interaction between a glycopeptide and a phosphopeptide produces sPGPs that form nanoparticles, which transform to nanofibrils upon partially enzymatic dephosphorylation. The assemblies form dynamically and hierarchically in situ on the cell surface, and interact with extracellular matrix molecules and effectively abolish contact inhibition of locomotion (CIL) of the cells. Integrating molecular recognition, catalysis, and assembly, these active assemblies act as a dynamic continuum to disrupt CIL, thus illustrating a new kind of biomaterials for regulating cell behavior.

Playing multifarious roles in cell organization, extracellular matrix (ECM) interacting with cells in 3D environment differ fundamentally from in 2D.[1] The elucidation of cell adhesion molecules has inspired attaching cell adhesion sequences (e.g., Arg-Gly-Asp (RGD)) to solid surfaces,[2] polymeric materials,[3] or supramolecular materials[4] for 2D or 3D cell culture.[5] However, two important post-translation features of ECM, glycosylation and phosphorylation, are largely overlooked. Every living cell is covered with complex array of glycans;[6] many proteins that associate with cell-matrix adhesion are phosphorylated[7] or dephosphorylated dynamically. Therefore, biomaterials acting as an artificial microenvironment for guiding cells must mimic the functions of glycoproteins and phosphoproteins.[8] But the synthesis of glycans remains a daunting challenge,[9] and the controlled protein phosphorylation is hardly an easy task. Thus, it is imperative to develop a facile approach for generating next generation biomaterials that mimic the functions of extracellular glycoproteins and phosphoproteins.

To sidestep the difficulty of synthesizing glycoproteins and phosphoproteins, we chose to generate higher-order assemblies of supramolecular phosphoglycopeptides (sPGPs, Fig. 1A) as their functional mimics because (i) higher-order assemblies of proteins, with a structural and dynamic continuum, are emerging as a new paradigm in biology to understand cellular functions,[10] but these insights have yet to be applied in designing the next generation biomaterials; (ii) assemblies of small molecules in the cellular milieu exhibit emergent properties.[11] To verify the roles of saccharides and dephosphorylation, we intentionally excluded known cell adhesion sequences in the phosphopeptides or the glycopeptides. We used the ligand-receptor interaction[12] (Fig. 1B) between a phosphopeptide (**1P**) and a glycopeptide (**2**) to form a sPGP (**1P:2**), which self-assembled to form oligomers (e.g., **(1P:2)6**, Fig. 1C) and existed as nanoparticles with diameters of 8±2 nm (Fig. 1D). Adding alkaline phosphatase (ALP)[13] to the solution of **1P:2** turned the nanoparticles into nanofibrils with diameters of 8±2 nm (Fig. 1D). Liquid chromatography–mass spectrometry (LC-MS) revealed that the assemblies of **1P:2** were partially dephosphorylated (Fig. 1E). Circular dichroism (CD) spectra (Fig. S1) of enzymatic dephosphorylation of **1P** exhibited a positive peak of 192 nm and a negative peak around 212 nm, suggesting a β-sheet arrangement. Mixture of **1P** with **2** yields the assemblies adopting the conformation that similar with **2**. Being partially dephosphorylated by ALP, **1P:2** yields the assemblies slightly increasing the signal of CD spectra, evidenced by two negative peaks at 200 nm and 212 nm. These results confirm that the assemblies of **1P:2** are dynamic (i.e., reversible binding between **1P** (or **1**) with **2**) and reactive (i.e., as substrates of phosphatases), thus generating a dynamic continuum of the assemblies consisting of peptide, phosphopeptide, and glycopeptide (Fig. 1A). Such dynamic assemblies are particularly important for functionally mimicking ECM because the ECM components themselves are oligomeric, multifunctional, and dynamic.

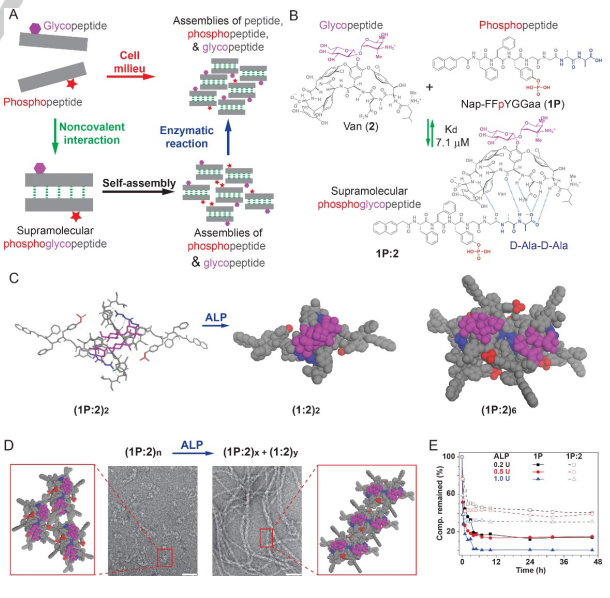

Figure 1. (A) Illustration of sPGPs, formed by non-covalent interaction, as a dynamic continuum in cell milieu. (B) Ligand-receptor interactions (green dash lines, dissociation constant (Kd) is 7.1 μM)) between vancomycin (Van, **2**) and the D-Ala-D-Ala containing phosphopeptides (Nap-FFpYGGaa, **1P**) result in a

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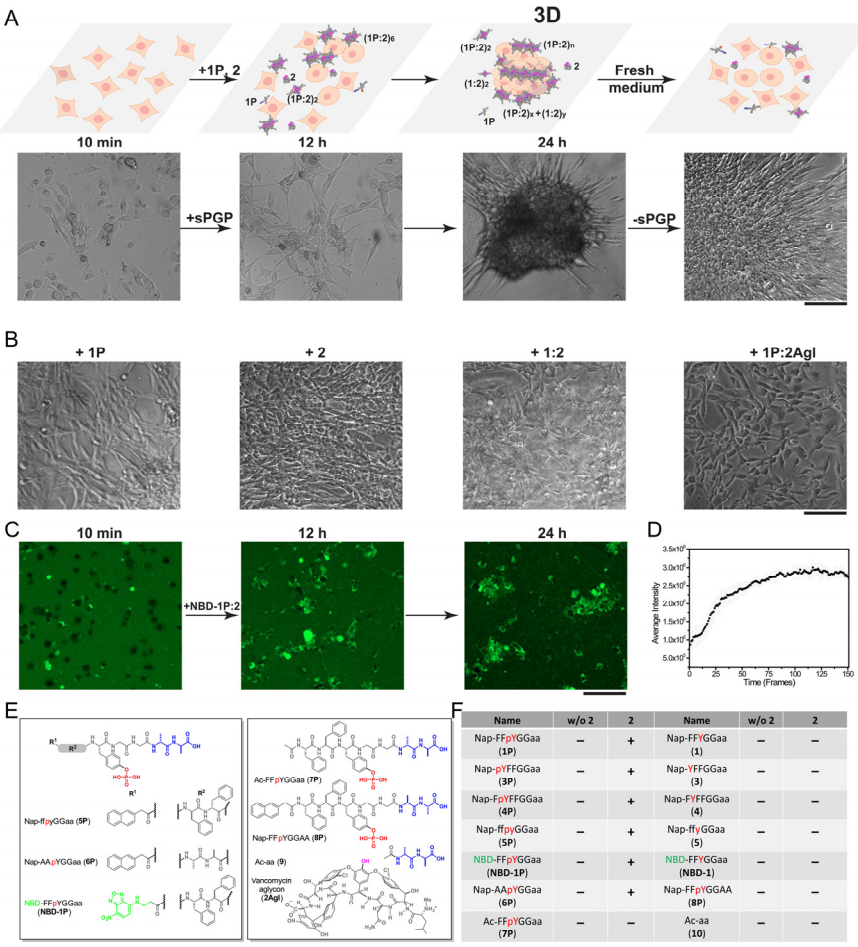
sPGP **(1P:2)**. (C) Enzymatic conversion of a dimer of the sPGP **((1P:2)$_2$**, stick model) to a dimer of supramolecular glycopeptide **((1:2)$_2$**, CPK model)), and the CPK model of a hexamer of **1P:2**. The dimer and hexamer are derived from the known crystal structure.[14] (D) Transmission electron microscopic (TEM) images of **1P:2** (300 μM) before (left) and after (right) being treated by a phosphatase (ALP, 1 U/mL. 24 h). Scale bar is 20 nm. (E) Phosphatases (ALP, 0.2, 0.5, and 1 U/mL) catalyzed conversion of **1P** (300 μM) and **1P:2** (300 μM) within 48 h.

To test if the designed assemblies functionally mimic ECM, we used spheroid formation by a fibroblast cell line (HS-5[15]) as a simple assay (Fig. 2A), for several reasons: (i) Fibroblast synthesize 3D ECM slowly (i.e., requiring days)[1]. Thus, if the cells rapidly switch from a 2D sheet to 3D spheroids, the assemblies likely act as ECM. (ii) Changing from 2D to 3D, cells have to remodel ECM so the assemblies must be adaptive and dynamic. (iii) In vitro assay reduces the cost and the complexity of in vivo models. (iv) Most importantly, the addition of phosphoproteins (e.g., FGF2) or glycoproteins (e.g., TGF-β1) induces clustering of HS-5 cells.[16] Thus, the assemblies of the sPGPs, if acting as functional mimics of glycoproteins and phosphoproteins, should induce 3D spheroids of HS-5 cells. Live imaging confirmed that, after being incubated with **1P:2** for 24 h, the HS-5 cells migrate and arise from surface to form 3D cell spheroids (Fig. 2A, video S1). In controls, HS-5 cells, without any treatment, remained as a 2D cell sheet (Fig. S2), likely due to contact inhibition of locomotion (CIL) (video S2).[17] The 3D spheroids spread to revert back to the 2D cell sheet after the removal of **1P:2** (Fig. 2A), indicating that the assemblies maintain the 3D spheroids. As shown in Fig. 2B, adding **1P** or **2** alone into the culture of HS-5 cells failed to lead to 3D spheroids. Neither the completely dephosphorylated complex **(1:2)** nor the complex of **1P** with vacomycin aglycon **(1P:2Agl)** induced cell spheroids. Co-incubation the cocktail of phosphatase inhibitors[18] with **1P:2** prevented formation of cell spheroids (Fig S4). These results confirm that the phosphotyrosine on the peptide (**1P**), the glycoside on **2**, and the in-situ dephosphorylation reaction are indispensable for inducing 3D spheroids.

We introduced a nitrobenzofurazan (NBD)-capped phosphopeptide (**NBD-1P**) because NBD fluoresces intensely when forming assemblies.[19] Adding **NBD-1P:2** into the HS-5 cell culture induced the cell spheroids after 24 h (Fig. S5). Live cell imaging (video S3) showed that the fluorescence increased gradually, indicating that more assemblies of **NBD-1:2** formed upon dephosphorylation (Fig. 2C) over time, as well as suggesting the formation of intermediate structures. The fluorescence on the cells (Fig. 2D) and the size of the cell spheroids increase simultaneously, indicating that assembling of **NBD-1:2**, indeed, is an in-situ, dynamic process. Meanwhile, the HS-5 cells treated by only **NBD-1P** or by **NBD-1:2** (Fig. S6) exhibit little fluorescence and hardly form spheroids. These results confirm that the reactive **sPGP** assemblies act as a dynamic continuum of nanostructures to induce 3D spheroids.

We synthesized the analogs of **1P** and mixed each of them with **2** for the cell assay. Scrambling the sequence of **1P** results in **3P** and **4P**, and introducing D-phenylalanine and D-tyrosine generates **5P**. **3P:2**, **4P:2**, or **5P:2** induced the cell spheroids while **3P**, **4P** or **5P** alone was ineffective, indicating that the regiochemistry or stereochemistry of the FFY segment in **1P** hardly affected the functions of the dynamic assemblies. Replacing the diphenylalanine by dialanine yields **6P**, and **6P:2** only induced the HS-5 spheroids slowly (after 48 h incubation). The acetyl-capped phosphopeptide (**7P**) was unable to self-assemble after dephosphorylation (Fig. S8), and **(7P:2)** failed to induce the cell spheroids. Using L-Ala-L-Ala to replace D-Ala-D-Ala produced **8P**, which was unable to form sPGP and failed to induce cell spheroid in the presence of **2**. These results confirm that the self-assembling ability of the sPGPs and the formation of sPGPs are the prerequisites for inducing cell spheroids. Moreover, adding D-Ala-D-Ala, which competitively bound to **2** in the presence of **1P** and led to more long nanofibrils (comparing to without D-Ala-D-Ala), resulted in more pronounced formation of cell spheroids (Fig. S10). The activities of these analogs, as the molecular validation, confirm that in-situ, hierarchical, and dynamic assembling of sPGP enables 2D to 3D cell organization.



Name	w/o 2	2	Name	w/o 2	2
Nap-FFpYGGaa (1P)	–	+	Nap-FFYGGaa (1)	–	–
Nap-pYFFGGaa (3P)	–	+	Nap-YFFGGaa (3)	–	–
Nap-FpYFFGGaa (4P)	–	+	Nap-FYFFGGaa (4)	–	–
Nap-ffpyGGaa (5P)	–	+	Nap-ffyGGaa (5)	–	–
NBD-FFpYGGaa (NBD-1P)	–	+	NBD-FFYGGaa (NBD-1)	–	–
Nap-AApYGGaa (6P)	–	+	Nap-FFpYGGAA (8P)	–	–
Ac-FFpYGGaa (7P)	–	–	Ac-aa (10)	–	–

Figure 2. In-situ, dynamic assembling of sPGPs transforms a cell sheet to cell spheroids. (A) The illustration of forming 3D spheroids from a 2D cell sheet upon the addition of sPGP and the reversibility of the process. (B) HS-5 cells only form 2D sheets when being co-cultured with phosphopeptide (**1P**), glycopeptide (**2**), **1:2** or **1P:2AgI** (vancomycin aglycon), respectively, for 48 h. Scale bars (in A, B, and C) are 50 μm. (C) Fluorescence changes during the formation of cell spheroids induced by **NBD-1P:2**. (D) Change of fluorescence on HS-5 cells treated with **NBD-1P:2** within 24 h at λex = 488 nm (three repeats). (E) Structures of the analogs of **1P**. (F) Summary of the states of the HS-5 cells co-cultured with different analogs of **1P** without or with **2**. All molecular concentrations are 300 μM.

Since the sPGP assemblies and the cell spheroids formed simultaneously, we examined whether the assemblies interacted with the components of ECM or cell adhesion molecules. Immunofluorescence staining reveals that fibronectin, laminin, collagen III, and collagen IV partially co-localize with the assemblies of **NBD-1P:2** (Fig. 3A, videos S4 to S7). Mβ-CD, a disruptor of lipid rafts,[20] hardly prevents formation of the cell spheroids or the co-localization of fibronectin with the assemblies of **NBD-1P:2** (video S8), further confirming that the interaction between the assemblies and ECM components (independent from plasma membrane) is critical for forming cell spheroids. Notably, the morphology of fibronectin in the cell spheroids is long, fibrous (Figs. 3A and S11), differing from the short puncta in the control (Fig. S11). Since ECM dynamics involve adhesion molecules,[21] we also examined the localization of α_5 and β_1 integrins, which are major fibronectin receptors, and observed little overlap between these integrins and the assemblies (Fig. 3B and S13, videos S9 and S10). Antibodies against $\alpha_v\beta_3$ and β_3 integrins, receptors for vitronectin and a distinctive type of cell-matrix adhesion, hardly co-localize with the assemblies (Fig. S13, videos S11 and S12). These results exclude the direct interactions between the assemblies and integrins. Furthermore, immunofluorescent staining shows no co-localization of E-cadherin, N-cadherin, or E-selectin with the assemblies of **NBD-1P:2** (Fig. S13, videos S13 to15)). Moreover, antibody blocking by antagonistic monoclonal antibody (mAb) to β_1 integrin, E-cadherin, N-cadherin, or E-selectin is unable to prevent **1P:2** to induce the cell spheroids (Fig. S14). Western blot (Fig. 3D) indicates that the expression level of β_1 integrin, E-cadherin, N-cadherin, as well as FGF2 or TGF-β1[16], remains constant. Collectively, these results confirm that the dynamic assemblies of the sPGPs interact with ECM components (Fig. 3E) and robustly induce formation of cell spheroids.

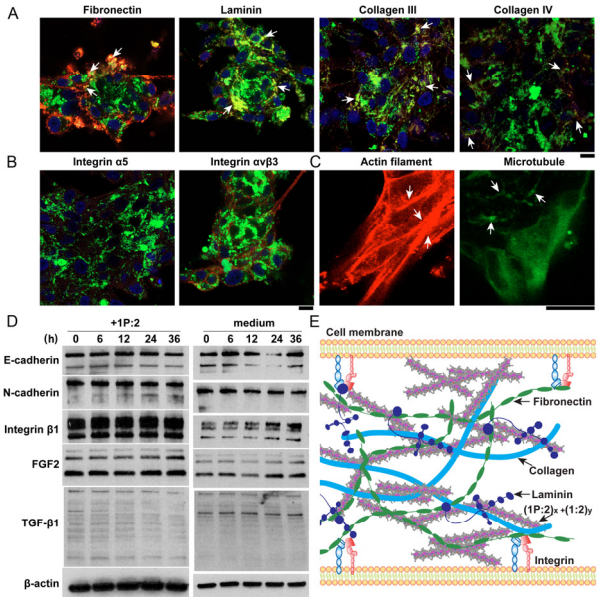


Figure 3. sPGP assemblies interact with ECM molecules but not the adhesion proteins. Immunofluorescence staining of HS-5 cells treated with **NBD-1P:2** (300 μM) for 48 h: the cell spheroids stained with antibodies of (A) ECM molecules (i.e., fibronectin, laminin, collagens III and IV) and (B) adhesive molecules (i.e., integrin α5, and integrin αvβ3). Green indicates the assemblies, red the antibodies, and blue the nuclei. Arrows point to the co-localization of the assemblies and the antibodies. (C) Morphology of cytoskeleton proteins in the HS-5 cell spheroids induced by **1P:2** (300 μM, 48 h). Actin cytoskeleton was stained with rhodamine-phalloidin (red), and microtubule with tubulin tracker (green). Arrows in actin and tubulin stainings indicate the fibrillar adhesion the reorganization of microtubule, respectively, in the cell spheroids. Scale bar in (A), (B) and (C) is 50 μm. (D) Time-dependent Western blot of HS-5 cells without or with the treatment of **1P:2** (300 μM). (E) Illustration of sPGP assemblies interacting with major ECM components.

We also examined the changes of F-actin filament and microtubules because cell migration and adhesion required the coordinated crosstalk between cytoskeletons.[22] Strong fluorescence of F-actin presents at the intercellular boundary in the spheroids, while weak fluorescence at the cell leading edge (Fig. 3C, video S16). In contrast, the phenomenon is reversed in cells without addition of the sPGP (Fig. S15). Since the intensity of fluorescence represent local actin polymerization[17] in the process of CIL, the intensity increase at crests of ripples traversing the leading edge (Fig. S15) is the common phenomena. However, in the cell spheroids, the intensity increase at the intercellular boundary, confirming that the adhesion sites and cell migration modes differ from the 2D cell movement.[5] Similarly, bright fluorescence of microtubule exists at the edge of the spheroids, while little cluster like fluorescence at the intercellular boundary (Fig. 3C, video S17). In the control HS-5 cells, the microtubules extend through the cell bodies (Fig. S15). These results suggest that the dynamic assemblies of sPGPs on the cell membrane provide additional forces to affect the actin (and tubulin) polymerization machinery at the leading edge, which alter migratory behavior of the cells and abolish CIL.

In conclusion, fundamentally differing from using cell adhesion sequence or 3D scaffolds, this work demonstrate the use of in-situ reaction and assembly as a spatiotemporal control to achieve 3D cell organization. It not only validates the dynamic continuum of the assemblies of small molecules as dynamic biomaterials to control cell behaviors for potential applications such as tissue engineering and wound healing, but also provides a simple model for studying collective migration of cells.

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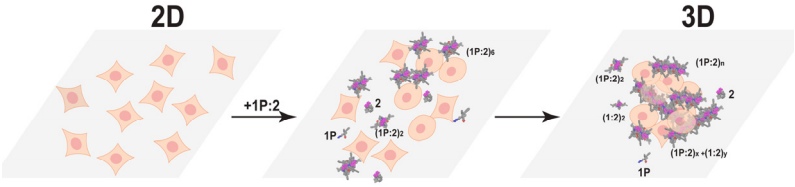

Enzymatically formed dynamic biomaterials in cell milieu modulate intercellular interactions to generate 3D cell spheroids.

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