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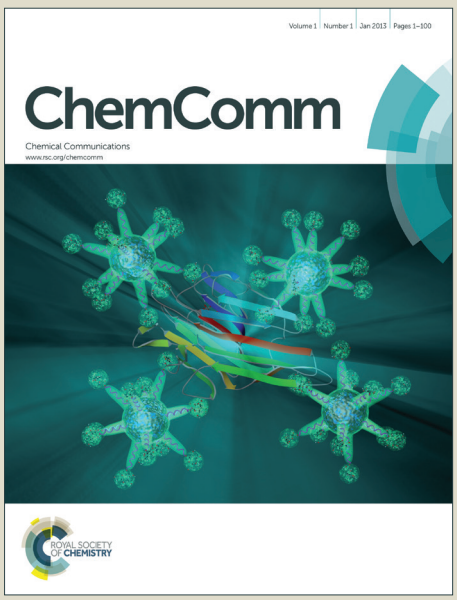





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# In Situ Growth of Polyphosphazene Nanoparticles Coating on Honeycomb Surface: Facilely Forming Hierarchical Structure for Bioapplication

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**Combined with breath figure method, an in-situ growth approach of polyphosphazene was performed on honeycomb surface resulting in nanoparticle close-packing coating and micro/nanoscale hierarchical structure. The hierarchically structured surfaces exhibited high biocompatibility, allowed good cellular adhesion and presented strong potential use as cell scaffold.**

Artificial scaffolds, which are designed to support cell adhesion and tissue growth, have been focused on the biomimic fabrication of hierarchical topographies, because cells are sensitive to the surface topography, at both microscale and nanoscale.[1-5] In general, the micro-topography affects the whole cell morphology and the nanoscale structures influence the adhesion of cells by Cell-extracellular cell matrix (ECM： ligand-antigen) interaction via filopodia microspike or membrane receptors.[6, 7] *In vivo*, the cellular adhesion and even the formation of tissue usually rely on hierarchical structures. For example, bone is hierarchical organization which composed of collagen protein and inorganic compounds with different scales structures.[8-11] Thus, a facile fabrication process of structures with multiple scales and tailored features would be more interesting and closer to the cells physiological environment than those with only monoscale ones.[12]

In order to further meet the growth of cells for the tissue engineering, in the past decade, various surface topography features, including nanowires arrays, microgrooves, micro pillars and pores, obtained by chemical etching, microcontact printing or dip-pen lithography have been widely reported.[13-15] Most of them were laboriously prepared and/or relied on a specialized template，and these materials with features at both micro/nano scales were inadequate for cell application at a large scale.[16, 17] Thus, developing a hierarchical cell scaffold is still intensively required. In addition, it is also a challenge to achieve desired organization with tunable structural parameters.

Recently, a self-assembly method called breath figure is utilized to prepare ordered hexagonal porous array, so-called “honeycomb structure”.[18-20] Since the pioneer work by Boker in the fabrication of hierarchical structure that the walls of honeycomb cavities were decorated with CdSe nanoparticles via pre-doping the nanoparticle into the polymer solution, the honeycomb porous surfaces have been used as macrostructure in many hierarchically structured systems.[21, 22] Among them, the used nanomaterials included carbon nanotubes, nanoclay, metals and metallic oxides, and other inorganic compounds.[23-25] It is worth to note that in the approach, the nanomaterials need to be pre-doped and stabilized by surfactants in polymer solution when performed a breath figure process. For the accumulation of nanoparticles on water droplet surface in emulsion, accurate interface tension and appropriate surfactants need to be carefully designed, the diameters of nanoparticles were limited as well.[26] What is more, the surfactants or metal nanomaterials were undesirable in many bio-systems.[27-29] A potential strategy to alter this situation is to grow biocompatible nanostructure in situ from the honeycomb surface. However, up to now, the strategy was not reported.

Phosphazene polymers, consisting of a backbone of alternating phosphorus and nitrogen atoms, exhibit high thermal stability, mechanical strength, biocompatibility and presents great potential as elastomers, fire retardants, solid battery electrolytes, and biomaterials.[30-33] However, there are still few polyphosphazene commercial products due to the laborious synthesis of linear polyphosphazene via ring-opening polymerization and macromolecular substitution approach or living cationic condensation process.[31, 34] Even though a one-step precipitation polymerization method can be used to prepare cyclo-matrix polyphosphazene. The obtained powder-like products, such as microspheres or micro-tube, limited their applications only as drug-release devices or bioimaging label.[30, 35, 36] Up to now, polyphosphazene surface via this simple method has not been reported. In this communication, the polyphosphazene nanoparticles (PNPs) coating with particle-pebbling structure were designed to grow in situ from the microscale polystyrene honeycomb films (PSHCF). It provides not only a facile method to prepare the phosphorus-containing surface but also a new way to gain micro-nano hierarchical structure. In detail, PSHCF was prepared via standard breath figure process (Figure S1) and the honeycomb hole was controllable.[37] On PSHCF, the diameter, density and location of PNPs were modulated. Furthermore, this hierarchical surface exhibited high bio-compatibility for cell adhesion.

The schematic diagram of the preparation process of PNPs decorating PSHCF is shown in Figure 1a. The hydrophobic PSHCF and the hydrophilic plasma-treated PSHCF were subsequently

immersed into the ethanol solution of a phosphonitrilic chloride trimer (HCCP) and 4,4-Dihydroxydiphenylsulfone (BPS), where triethylamine (TEA) was added as acid-binding agent. PNPs grew step by step on the hydrophobic surface and finally formed a PNPs pebbling PSHCF. While on the hydrophilic surface, the PNPs escaped directly from the surface. Consequently, PNPs failed to decorate the PSHCF surface.

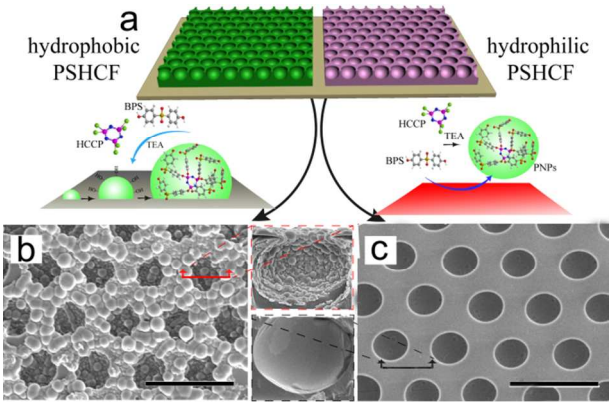


**Figure 1**. a) Schematic of the process of decoration of PSHCF with polyphosphazene: PNPs are grown in situ on the original, hydrophobic PSHCF surface. PNPs are unable to grow on plasma-treated PSHCF (hydrophilic). b) SEM image of hydrophobic PSHCF decorated with PNPs. The cross section shows that both exterior and interior of the honeycomb was decorated with PNPs. Scale bar = 10 μm. c) SEM image of hydrophilic PSHCF decorated with PNPs. PNPs (a cross section of which is shown in the inset) cannot grow on the surface. Scale bar =10 μm.

Scanning electron microscopy (SEM) was employed to characterize the morphology of the obtained ordered hierarchical structure. As shown in Figure 1b, the microscale honeycomb pores have been remained, and a confluent layer of PNPs covering the surface of the honeycomb surface can be observed. Furthermore, cross-section SEM photographs of the pore showed that a uniform PNPs layer formed on the inner surface of honeycomb holes as well. Significantly, the nanoparticles were observed to be semi-spherical shape rather than whole sphere, resembling pebbles. Such a pebble structure indicated the intensive affinity between nanoparticles and nanoparticles as well as nanoparticles and surface. The pebbling structure is also attributed to that the PNPs grew in situ from the surface rather than just adsorbed after the formation of PNPs. The surface energy or the wettability of honeycomb surface was considered to be of the vital importance for the in-situ growth of PNPs. We tried to modulate the PSHCF surface into hydrophilicity with a water contact angle (WCA) of 30 ° by plasma treatment at 100w for 3 min. After in-situ polymerization, Figure 1c exhibited none PNPs decoration on the hydrophilic PSHCF. In this case, the cavities of honeycomb structure also showed non-decoration with PNPs (Figure 1c insert). The cross-section SEM photograph provided the smooth inner honeycomb wall because of no pebbling when the surface was hydrophilic.

From the viewpoint of solubility parameter theory, the stability of interface between two polymers is not only affected by the individual intrinsic physical properties but also vitally decided by the interaction between them.[38-40] Thus, for the deposition of PNPs on PSHCF surface, good match in surface energy between the two polymers should be taken into account. The surface of PS is hydrophobic and has strong affinity toward a surface of similar surface energy.[41, 42] The surface energy was 43.17 $mN.m^{-1}$ for PSHCF and 40.11 $mN.m^{-1}$ for PNPs (Figure S2).[43] Owing to the matched surface energy, polystyrene surface exhibited intensively physical adsorption to polyphosphazene oligomer, resulting in the in situ growing of PNPs on the PSHCF surface. However, the surface energy of PSHCF increased to 72.55 $mN.m^{-1}$ after plasma treatment. The huge energy difference between the modified substrates and PNPs made polyphosphazene difficult to grow on the substrate. These phenomena can also be understood from the interaction between molecules of PS substrate and phosphazene. As shown in Figure S3, the repeated unit of polyphosphazene was guided to move close to the polystyrene as close as 3 Å from 10 Å (average distance of typical attractive intermolecular forces called van der Waals forces).[44] The approach of polyphosphazene to the hydrophilic or hydrophobic surface was simulated by Gaussian 09.[45, 46] The results indicate that more energy was required for polyphosphazene to approach the hydrophilic PSHCF surface than that to approach the hydrophobic surface (Table S1). Herein, from the molecular lever and interface energy, the interaction of PNPs and PSHCF surface would be modulated by changing the surface energy of PSHCF substrate.

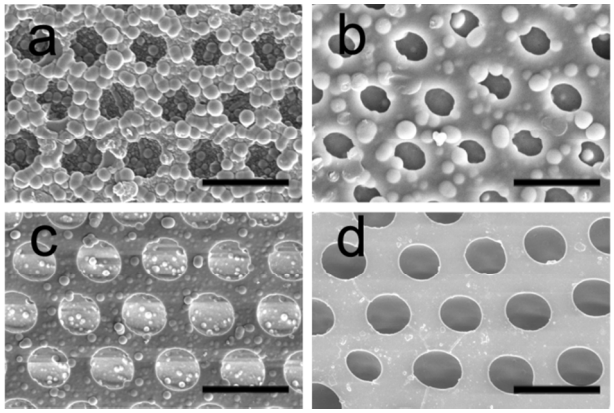


**Figure 2**. (a – d)SEM images of PSHCF decorated with PNPs. Modulation of the wettability of PSHCF substrates by plasma resulted in water contact angles of a) 110 °, b) 80 °, c) 60 °, and d) 30 °. Scale bar = 10 μm.

As a proof of concept, a series of PSHCFs with WCA at 110 °, 80 °, 60 °, and 30 ° were obtained by adjusting plasma treatment time, whose corresponding surface energies were 43.17, 52.06, 60.92 and 72.55 $mN.m^{-1}$ respectively. As shown in Figure 2a to d, with the improvement of hydrophilicity of PSHCF, the density of PNPs decoration decreased. On the PSHCF with WCA at 110 °, the polyphosphazene particles attached closely with each other and formed a confluent layer; but only a scattered distribution of the nanoparticles on PSHCF was described after WCA decreased to 80°, even scarce PNPs decoration when the PSHCF was turned into hydrophilic and none PNPs can be observed when the surface was totally hydrophilic (WCA=30 °). Hence, the density of PNPs can be controlled via tuning the wettability of PSHCF spanning from hydrophobic to hydrophilic.

Besides the surface wettability, the concentrations of monomers also play an important role in the growth process of PNPs. Here, the concentration of monomer HCCP was varied from 1-4 $mg.mL^{-1}$ with a molecular ratio of HCCP and BPS being fixed at 1:3. SEM was undertaken to observe the diameters of the PNPs. As depicted in Figure S4, the average size of nanoparticle increased from 180 to 1200 nm with the increase of concentration (Figure S4a to d). Therefore, by regulating the concentration of monomer, the diameter of PNPs had been successfully modulated. A brief summary of the density and size of nanoparticles is exhibited in Figure 3. Lower

substrate surface energy and higher monomer concentration facilitate the increase of density and size of nanoparticles.

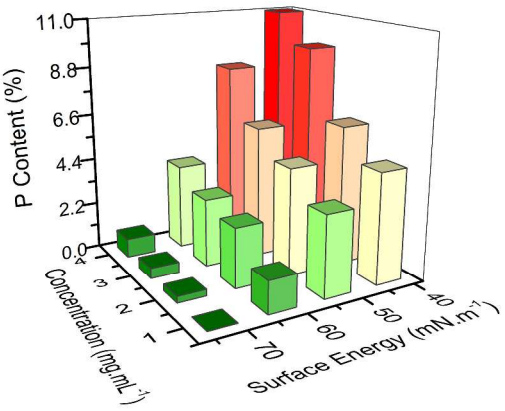


**Figure 3.** The relationship of polyphosphazene nanoparticles content (in weight, based on the EDS analysis) with concentration of monomers and the surface energy of polystyrene substrate in a 3-dimontional bar figure.

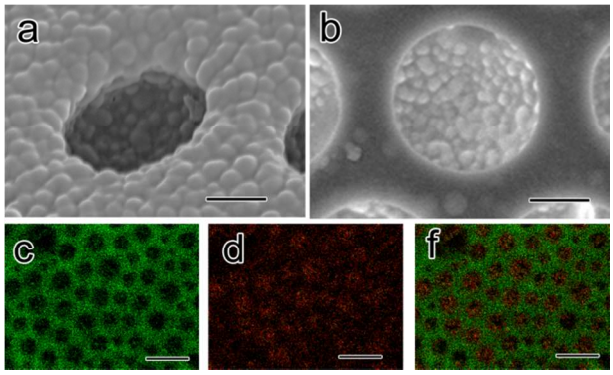


**Figure 4.** a) The inclined SEM of unselective patterned surface, b) the SEM images of patterned surface. Scale bar= 2 μm. c, d) carbon and phosphorous EDS maps of PSHCF selectively decorated with PNPs. f) Overlay of images in panels c and d: green and red areas represent carbon and phosphorous, respectively. Scale bar= 10 μm.

Apart from the density and diameter of PNPs, the patterned location of PNPs is also interesting. According to the above analysis, the PNPs tend to grow from a hydrophobic PSHCF rather than a hydrophilic one. We tried to obtain a selectively modified PSHCF with hydrophilic top surface and hydrophobic honeycomb pores via a rapid plasma treatment (15s) with a high intensity of $O_2$ plasma as high as 350 W. At the early stage of irradiation, the modified top surface of honeycomb acquired the same charge as the sheath of plasma. The strong mutual repulsion between two electric field shields stops further modification and protects hydrophobicity of honeycomb inner hole (Figure S5).[47] The inclined SEM of unselectively patterned polyphosphazene nanoparticles was showed at Figure 4a. Both the surface and the inner cavities of honeycomb structure were planted with polyphosphazene nanoparticle and the nanoparticles can contact with each other into a confluent layer. While on the patterned surface, only the holes of honeycomb structure were modified with the particles (Figure 4b). The distribution of polyphosphazene nanoparticles was also proved by map scanning and line scanning of energy dispersive spectrometer (EDS). As shown in Figure 4c-f, carbon mainly concentrated on the surface of PSHCF and phosphorous was patterned in the hole. The line scanning EDS spectra (Figure S6) showed alternating carbon and phosphorous peaks. The PNPs appeared in the pores and disappeared on the top surface of PSHCF, where the carbon atom arising, indicating that PNPs formed in the honeycomb pores based on selective modification of the surface wettability.

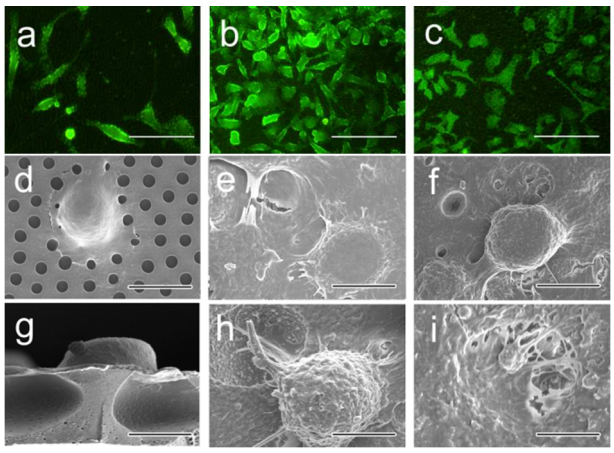


**Figure 5.** a-c) Double-staining fluorescence image of dead/live (EB/AO) HeLa cells, Scale bar=200 μm. d-f) SEM images of HeLa cells, Scale bar=20 μm. g-i) SEM images of HeLa cells, Scale bar=5 μm. PSHCF (column 1), PNPs covered PSHCF (column 2), PNPs selectively patterned PSHCF (column 3).

To investigate the potential application of polyphosphazene structured PSHCF, HeLa cells were cultured on PSHCF and PNPs decorated PSHCFs. The cells were seeded at $10^5$ cells/well in a 24-well plate containing the films and then cultured for 24 h. Cell imaging was performed by double staining of live and dead cells with acridine orange (AO) and ethidium bromide (EB), respectively. As shown in Figure 5a, few cells were on PSHCF but clearly increased in number on PNPs decorated PSHCFs (Figure 5b and c). As all cells on both films were stained green by AO, the surface exhibited high biocompatibility. Furthermore, CCK-8 assay was performed to detect the cell viability on PNPs decorated PSHCFs (Figure S7). Cell viability on all substrates reached 90%, indicating that PSHCFs decorated with PNPs were biocompatible for cell.
To acquire the morphological information of cells on the films, cells were fixed with glutaraldehyde and dehydrated with gradient alcohol. SEM images of cells on PSHCF and on PSHCF decorated with PNPs are displayed in Figure 5d to f. After 24 h of culture on PSHCF, the cells on PSFCF were nearly spherical (Figure 5d). However, cells on PNPs decorated PSHCFs exhibited a more spreading morphology: the cells formed visible lamellipodia, which almost connected with each other to form a confluent layer on the hierarchical surfaces (Figure 5e and f). This phenomenon indicates enhanced cell adhesion and cellular spreading. Interestingly, the cells on the PSHCF jumped over honeycomb hole (Figure 5g), while the viable filopodia of cells on the decorated PSHCF spread into pore (Figure 5h and i), which furthermore proved that the PNPs decorated PSHCF is an ideal cell scaffold material. With the culture time extending, the difference in cell number and spreading between the original PSHCF and PNPs decorated PSHCF became obviously lager (Figure S8 and S9). Therefore, apart from the sufficient biocompatibility from cyclomatrix polyphosphazene, the hierarchical structure with high porosity, high surface area and specific geometry, provided available space for cells to migrate and raised more cell-materials interaction. [48]

## Conclusions

In summary, we demonstrated a quite simple and facile method to prepare the cyclomatrix polyphosphazene coating. The polyphosphazene nanoparticles can be modulated and patterned to form hierarchical structures on honeycomb surface. This approach is unique in two aspects: 1) it can provide a rapid generation of phosphorus-containing hierarchical surface for cell scaffold; 2) the parameters of polyphosphazene coating were controllable without laboriously chemical modification. Moreover, it is not a polystyrene-specific method, which can also be applied to other substrates with matched surface energy. This polyphosphazene coating is potential to be used in the micro/nano interfaces or scaffolds.

## Notes and references

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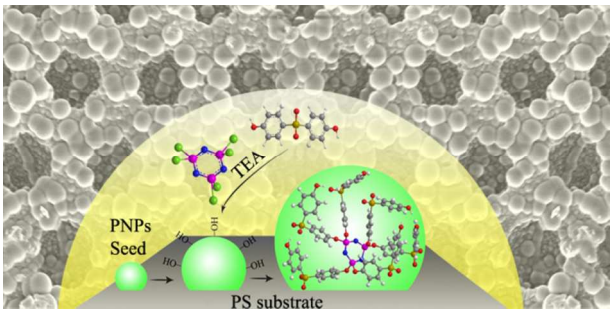
## *Table of contents*




Cyclomatrix polyphosphazene nanoparticles are targetedly in-situ grown from honeycomb surface for the preparation hierarchical cell scaffold.