Downloaded from orbit.dtu.dk on: May 04, 2024

DTU Library

Phosphocholine-Decorated PPI-Dendrimers Mimic Cell Membrane Phosphocholine Clusters and Tune the Innate Immune Activity of C-Reactive Protein

Boas, Ulrik; Daoud, Mohammad Mahdi; Meier, Sebastian; Olsen, Tobias Hegelund; Gehring, Kristoffer ; Juhl Mogensen, Ditte; Heegaard, Peter M. H.

Published in:
Biomacromolecules

Link to article, DOI:
10.1021/acs.biomac.1c00085

Publication date:
2021

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Boas, U., Daoud, M. M., Meier, S., Olsen, T. H., Gehring, K., Juhl Mogensen, D., & Heegaard, P. M. H. (2021). Phosphocholine-Decorated PPI-Dendrimers Mimic Cell Membrane Phosphocholine Clusters and Tune the Innate Immune Activity of C-Reactive Protein. *Biomacromolecules*, *22*(4), 1664–1674. https://doi.org/10.1021/acs.biomac.1c00085

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Phosphocholine-Decorated PPI-Dendrimers Mimic Cell Membrane Phosphocholine Clusters and Tune the Innate Immune Activity of C-Reactive Protein

Ulrik Boas[1]*, Mohammad Mahdi Daoud[1], Sebastian Meier[2], Tobias Hegelund Olsen[1], Kristoffer Gehring[1], Ditte Juhl Mogensen[1], Peter M. H. Heegaard[1,3]

[1]Innate Immunology Group, National Veterinary Institute, Technical University of Denmark, Lyngby, Denmark

[2]Department of Chemistry, Technical University of Denmark, Lyngby, Denmark

[3]Innate Immunology Group, Department of Biomedicine and Biotechnology, Technical University of Denmark, Lyngby, Denmark

*corresponding author, uboas@yahoo.com

Abstract:

C-reactive protein (CRP) is a widely used as biomarker of infection and inflammation. It has a well-described ability to bind phosphocholine (PC), as well as PC-clusters from compromised and inflamed cell membranes and tissues. The binding of PC-clusters to CRP is of interest as this binding determines subsequent innate immune activity. We investigated PC-decorated dendrimers as mimics for PC-clusters. Five generations of poly(propylene imine) (PPI) dendrimers were modified with PC surface groups via a three-step synthetic sequence obtaining the PC decorated dendrimers in high purity. The dendrimers were analyzed by NMR and infrared spectroscopy as well as HPLC. We developed immunoassays to show that dendrimer-PC binding to CRP was Ca^{2+}- dependent with an apparent overall Kd of 11.9 nM for first generation (G1) PPI-PC, while G2- and G3-PPI-PC had a slightly higher affinity, and G4-PPI-PC and G5-PPI-PC a slightly lower affinity. For all PC-dendrimers the affinity was orders of magnitude higher than the affinity of free phosphocholine (PC), indicating a PC-cluster effect. Next we investigated the binding of CRP:PPI-PC complexes to complement component C1q. C1q binding to CRP was dependent on the generation of PPI-PC bound to CRP, with second and third generation PPI-PC leading to the highest affinity. The dendrimer-based approach to PC-cluster mimics and the simple binding assays presented here hold promise as tools to screen PC-compounds for their ability to tune the innate immune activity of CRP.

Introduction

A large variety of medical conditions correlate with changed levels of inflammatory biomarkers such as acute phase proteins in serum. C-reactive protein (CRP) is a serum acute phase protein and is found in elevated concentrations as a response to inflammation and physical trauma.[1] In humans, slightly increased levels (3-10 mg/L) of CRP in serum have been found to be associated with a number of severe medical conditions such as ischemic heart disease and certain types of cancer.[2-4] CRP binds phosphocholine (PC),[5] a small molecule found as a sub-structure of phosphatidylcholine in cell membranes. Furthermore, PC is found as a substructure in a number of bacterial glycolipids and capsular polysaccharides and as a constituent of certain bacterial cell membrane types.[6] CRP is pentagon-shaped and comprises five identical subunits.[7] Each subunit is capable of binding one PC molecule with a dissociation constant in the micromolar range[8] subject to the simultaneous binding of two Ca^{2+} ions in the same subunit.[9] The CRP pentamer can therefore be regarded as a pentavalent receptor for molecular structures containing PC ligands, where the five identical recognition sites for PC enable CRP to bind strongly to its target ligand. Early work on monomeric PC derivatives showed that PC phosphodiesters had a significantly reduced CRP-binding compared to the PC monoester itself[10] whereas naturally occurring PC phosphodiesters such as phosphatidylcholine/lyso-phosphatidylcholine bind CRP with high affinity provided they are made accessible by disrupting the lipid bilayer cell membranes, in which they reside.[11] Also, certain synthetic constructs employing PC diesters can bind CRP with high affinity.[12] The common feature of PC diesters binding CRP with high affinity is that they are all multimeric, either organized as PC clusters in natural systems or being constituents of chemical polymers in artificial systems.

Phosphocholine binding takes place at one face of the CRP pentamer (the 'B face'), whereas the opposite face of the protein (the 'A face') is the effector face (Figure 1). The effector face is involved in immune activation, binding the complement factor C1q and Fcγ receptors of innate immune cells (Figure 1)[13] promoting phagocytosis of the target cell to which CRP is bound as well as inducing release of proinflammatory cytokines for further immune activation. Binding of sufficient numbers of PC moieties at the B face leads to a conformational change which increases the affinity for complement factor C1q at the A face of the CRP pentamer (Figure 1).[9] Thus, the oligomeric state of the PC ligand is suggested to play a key role in modulating the innate immune activity of CRP. This was shown in a number of early papers utilizing natural but molecularly undefined membrane- and liposome-derived PC clusters.[14, 15]

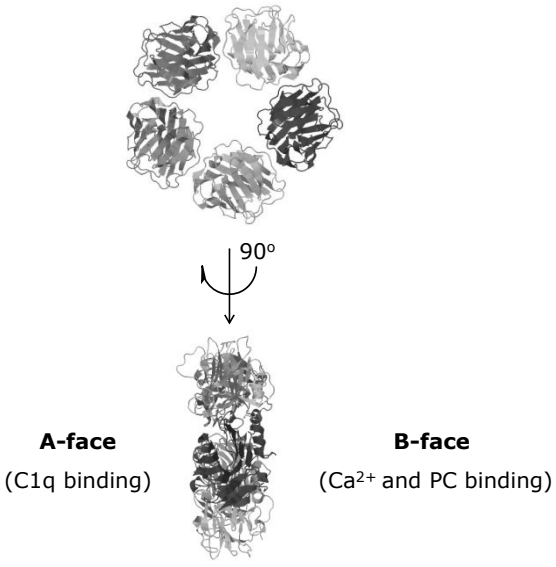


Figure 1. Top: The pentameric and pentavalent structure of human C-reactive protein (CRP). Bottom: Side-view of CRP indicating the phosphocholine/calcium ion binding face (B face) and the C1q/neutrophil effector face (A face).[16] The figure is based on the RCSB PDB entry 1GNH rendered in JSmol.

While PC is present as multimers ('PC clusters') in biological systems, only few studies describe the relation between the structure of PC clusters and the binding and activation of CRP. Synthetic, molecularly defined minimal PC clusters (bis-phosphocholine compounds) were investigated by Pepys et al. [17] and were demonstrated to cross-link two native CRP pentamers blocking rather than promoting CRP activation. However the effect of high-affinity multivalent binding of molecularly defined synthetic PC clusters with other dimensions and higher valency on the biological activity of CRP has not been investigated previously. We therefore initiated studies to investigate the binding of well-defined oligomeric PC ligands by CRP and how this binding affects the interaction with complement factor C1q. For this purpose, the synthesis and use of PC-decorated poly (propylene) imine (PPI) dendrimers acting as well-defined multivalent PC cluster mimics were investigated. Dendrimers are synthetic monodisperse hyperbranched polymers whose molecular size increases in a defined manner with increasing dendrimer generation, the generation number being defined as the number of branching points from the core to the periphery (see Figure 2).[18] The number of surface groups will double with each generation, from a G1-PPI dendrimer with 4 surface groups to G5-PPI having 64 surface groups.

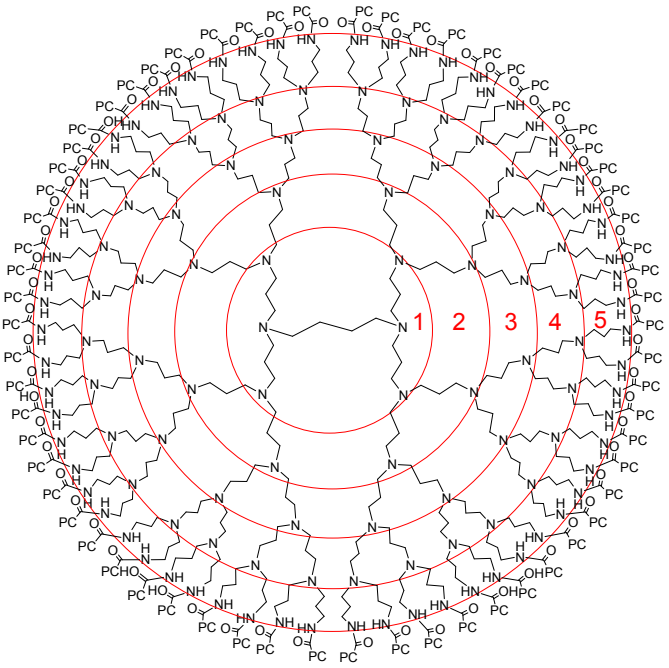


Figure 2. Structure of the PPI dendrimer scaffold carrying PC surface molecules. The circles and numbers denote dendrimer generations. Each generation increases the size and multimericity of the dendrimer.

For each generation of a PPI dendrimer, the density of the surface groups increases, and the dendrimer reaches dimensions of several nanometers. Dendrimers have been investigated as potential scaffolds for biomedical applications.[19] Especially the multivalency of dendrimers has attracted ample interest, for instance in dendrimers with carbohydrates at their surfaces showing improved binding of bacterial lectins and adhesins such as the lectin Concanavalin A,[20] Shiga-like toxin[21, 22] and FimH[23]. In these systems, it was found that when the dendrimer generation is increased to a certain extent, the affinity of the multivalent dendrimer-lectin/adhesin binding can be increased from several 100-fold to ten thousand-fold compared to the monomeric carbohydrate. We have previously shown that multivalency of dendrimers can also lead to increased cytokine excretion from immune cells (peripheral blood mononuclear cells) upon interaction with myramyldipeptide (MDP) surface modified dendrimers in cell culture.[24] In the current study, the binding of CRP to novel types of PPI-

PC dendrimers and the resulting impact on the ability of CRP to bind the complement factor C1q was investigated, elucidating how manipulation of B face PC binding influenced C1q binding, thus potentially modulating the innate immune activity of CRP.

Experimental section

Materials and Methods

Chemical synthesis: Commercially available starting materials such as 2-methacryloyloxyethyl phosphorylcholine, methyl 3-mercaptopropionate, lithium iodide, oxalyl chloride, PyBOP, BTFFH and TFFH were purchased from Sigma-Aldrich (St. Louis, MO). PPI-dendrimers were purchased from SyMO-Chem BV (Eindhoven, The Netherlands), synthesized from a 1,4-diaminobutane (DAB) core and provided in generations 1-5. Solvents were purchased from Fischer Scientific (Fair Lawn, NJ) with HPLC grade purity. Preparative HPLC purification was carried out on a Shimadzu Prominence LC-20AP equipped with a SPD-20A detector (wavelength 190 nm) using a Supelco Ascentis C18, 25cmx10mm, 5µm and a 5mL/min linear gradient 0-15 min. (0-100% buffer B), 20-25 min. (100-0% buffer B), stop time 30 min. Buffer A: 0.025% TFA in 10% aqueous acetonitrile. Buffer B: 0.025% TFA in 90% aqueous acetonitrile.

Chemical characterisation tools: NMR spectra were acquired on dendrimer samples at natural isotopic abundance, dissolved in 600 µl d6-DMSO (Sigma-Aldrich) and transferred to 5 mm NMR sample tubes. Initial NMR spectra (1D ^{1}H, 1D ^{13}C and ^{1}H-^{13}C HSQC) were acquired on a 300 MHz Bruker Avance equipped with a BBO probe and an autosampler. DQF-COSY, TOCSY, NOESY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C standard and edited HSQC, ^{1}H-^{15}N HSQC as well as ^{1}H-^{13}C HSQC-TOCSY and ^{1}H-^{13}C HSQC-NOESY spectra were employed for spectral assignment and compound characterization and performed on an 800 MHz

Bruker Avance III HD spectrometer equipped with a TCI CryoProbe. NOESY and ^{1}H-^{13}C HSQC-NOESY spectra were acquired using mixing times of 150 milliseconds. ^{1}H-^{31}P HSQC spectra were acquired on a 600 MHz Bruker Avance III spectrometer equipped with a BBO Smart probe by recording 1024(^{1}H)×128(^{31}P) complex data points, sampling 131 milliseconds in the ^{1}H dimension and 18 milliseconds in the ^{31}P dimension, respectively. One-dimensional ^{31}P spectra were recorded by sampling 32k complex data points during an acquisition time of 340 milliseconds. A sweep width of 395 ppm was employed to observe residual PF_6^- ions at a chemical shift of -144 ppm. Overall, a reporter-group concept was applied to resolve amide bond formation upon phosphocholine attachment to the dendrimers and intact bonds (for instance phosphodiesters) in the phosphocholine upon coupling. All spectra were processed with extensive zero filling in all dimensions and analyzed using Bruker Topspin 3.5 pl5.

HPLC-MS analysis was carried out using a Shimadzu Nexera X2 HPLC connected with a Bruker MicrOTOF-Q III mass spectrometer, using a Phenomenex Kinetex C18 column (2.6 µ, 100 Å, 50×2.1 mm) and a 1 mL/min. linear gradient using 0-2.7 min. (0-20% buffer B), 2.7-4.5 min. (20-100% buffer B), 6-7 min. (100-0% buffer B), stop time 10 min. Buffer A: 0.025% TFA in 10% aqueous acetonitrile. Buffer B: 0.025% TFA in 90% aqueous acetonitrile. Size exclusion chromatography (SEC) data were obtained for all samples using a Shimadzu LC-2010A equipped with a Phenomenex BioSep-SEC-s2000 column (300 × 7.80 mm) under isocratic conditions (buffer 0.1% TFA in 30% aqueous ethanol) with a flow of 0.800mL/min, stop time 19 min. Samples were dissolved in Milli-Q water using Snap Seal™ vials (2 mL volume, 12 × 32 mm) provided by Sigma-Aldrich. Buffer mixture: 0.1% TFA in 50% aqueous EtOH. Infrared (FT-IR) spectra were obtained from small quantities of

dry samples (1-3mg) using attenuated technique of reflectance (ATR) for all samples on a Shimadzu IRAffinity-1 equipped with a MIRacle 10 ATR cell.

Methylester-PC (1): 2-methacryloyloxyethyl phosphorylcholine, MPC (3g, 10.2 mmol) and methyl 3-mercaptopropionate (1.72 mL, 13.2 mmol) were mixed together in ethanol (20 mL). N_2 gas bubbling was performed for about 15 min. Diisopropylamine (DIPA, 1.0 mmol (10 mol%), 74 µL) was added to the solution as a catalyst. After stirring for 20 h at room temperature, ethanol was evaporated under vacuum, and the residue was washed 3 times with ethyl acetate (EtOAc) and Milli-Q water to remove non-reacted compounds. The product partitioned in the water phase, and the by-product was in the EtOAc-phase. Finally, the water phase was separated and freeze-dried. Yield 4.14 g (98%). HPLC: One peak t_R 0.8-1.1 min, MS: Calcd. ($C_{15}H_{30}NO_8PS$) 415.16, found 416.17 ($[M+H]^+$. FT-IR (ATR) ν in cm^{-1} 771($(RO)_3$ P=O), 1080/1244 (C-O ester-bond), 1728 (C=O, ester). ^{1}H NMR (300 MHz, D_2O, 4.79 solvent peak) δ_H in ppm 1.28-1.30 (d, *J* 6.87 Hz, 3H, -CH_3), 2.74-2.84 (dd, 3H *J* 6.84 Hz(-CH_2-) and *J* 6.80 (-CH-)), 2.85-2.94 (mult, 4H, -CH_2-), 3.28 (s , 9H, N^+-CH_3), 3.70-3.74 (t, *J* 9.85 Hz, 2H, CH_2-N^+), 3.77 (s,, 3H, CH_3-O), 4.13-4.19 (dd, 2H, *J* 7.04 (CH_2-O-$P(O)_3$-) and *J* 7.04 (CH_2-O-$P(O)_3$-)), 4.32-4.40 (mult, 4H, $P(O)_3$-CH_2- and CH_2-O). ^{13}C-NMR (75 MHz, D_2O) δ_C in ppm: 16.2 (CH_3), 26.9 (-CH_2-S-), 34.2 (-S-CH_2), 34.5 (-CH_2-), 40.1 (-CH-), 52.3 (CH_3-O), 54.0 (CH_3-N^+), 59.4 (CH_2-O-P), 63.8 (CH_2-O-P), 64.5 (1C, CH_2-O-C=O), 66.0 (CH_2-N^+), 175.1 (C=O), 177.4 (C=O)

COOLi-PC (2): In a 100 mL reaction flask compound (**1**) (3.2 g, 7.7 mmol) and LiI (4.12 g, 30.8 mmol) were dissolved in dry tetrahydrofuran (THF) (25 mL). The mixture was gently refluxed overnight (oil bath temperature ca. 150^{o}C), while bubbling nitrogen gas through the solution to remove methyl iodide formed in the reaction. The solvent evaporated overnight, leaving an orange crystalline residue, which was triturated twice in acetone (10 mL) for 30

min to give the product as white crystalline. If there were impurities of unreacted starting material after the trituration, another trituration was performed under heating. Eventually, the residue was dissolved in methanol (MeOH), and then re-crystallised by dropwise addition to cold acetone (5 times MeOH volume) and the white crystalline precipitated in a purer form. Yield: 1.97 g (63%). HPLC: t_R Product peak 0.3-0.6 min, MS: Calcd. for COOH-PC ($C_{15}H_{27}NO_8PS$) 401.13, found 402.16 $[M+H]^+$ $[2M+H]^+$. FT-IR (ATR) ν in cm^{-1} 794 ($(RO)_3P=O$), 1090/1222 (C-O in ester), 1585/1411 (C=O, carboxylate), 1726 (C=O, ester), 3200-3400(O-H stretch). ^1H NMR (300 MHz, D_2O, 4.79 solvent peak) δ_H in ppm 1.25-1.27 (d, *J* 6.64 Hz, 3H, $-CH_3$), 2.45-2.49 (t, *J* 14.4 Hz, 2H, $C=O-CH_2-$), 2.71-2.93 (m, *J* 6.4 Hz, 5H, $-CH_2-S-$, -CH-), 3.24 (s, 9H, N^+-CH_3), 3.67-3.70 (t, *J* 9.24 Hz, 2H, $-CH_2-N^+$, 4.10-4.16 (dd, 2H, *J* 7.04 ($CH_2-O-P(O)_3-$) and *J* 7.04 ($R-CH_2-O-P(O)_3-$)), 4.30-4.38 (mult, 4H, $P(O)_3-CH_2-$ and $R-O-CH_2-$) ^{13}C NMR (75 MHz, D_2O) δ_C 16.5 (CH_3), 28.7 (CH_2-S), 34.6 (CH_2-S), 37.8 (CH_2), 40.4 (CH), 54.3 (CH_3-N^+), 59.8 (CH_2), 64.0 (CH_2), 64.7 (CH_2), 66.2 (CH_2-N^+), 178.1 (C=O), 180.9 (C=O).

G1-PPI-PC (3): Compound 2 (1.5 eq, 50 mg, 0.12 mmol) and TFFH (1.7 eq, 39 mg, 0.15 mmol) were dissolved in NMP (1 mL) and DIPEA (3 eq., 24.9 µL) was added to the solution and mixed for 15 mins for intermediate activation. PPI-G1 (1 eq., 7.8 mg, 0.025 mmol, end-group conc.: 0.098 mmol) was added and the reaction was stirred for 20 h at room temperature. Ninhydrin test was performed to check for reaction completion. If negative, the solution was slowly added dropwise to cold diethyl ether to give the product as white crystalline precipitate. The supernatant was decanted and the precipitate was washed three times with ethanol (4-5 mL) for 15 mins. The precipitate was dissolved in Milli-Q water and freeze dried overnight to give the product. Yield: 6.1 mg (13.5%). SEC: t_R 11.4 min. FT-IR (ATR) ν in cm^{-1} 791 ($(RO)_3P=O$), 1032/1219 (C-O, ester), 1651 (C=O, amide), 1726 (C=O,

ester). ^{1}H-^{13}C-HSQC NMR (800 MHz/200 MHz, d_6-DMSO) δ_H/δ_C in ppm 1.15/16.9 (CH_3), 1.71/20.9 (CH_2), 1.80/24.0 (CH_2), 2.41/36.0 (CH_2-C=O), 2.70/40.0 (CH-C=O), 2.72/28.0 (CH_2-S), 2.79/2.63/35.0 (CH_2-S), 3.0/51.7 (CH_2-N), 3.10/36.3 (CH_2-NH amide), 3.17/53.6 (CH_3-N^+), 3.57/65.7 (CH_2-N^+), 3.93/63.3 (CH_2-O-P), 4.13/59.1 (CH_2-O-P), 4.24/64.5 (CH_2-O-C=O). ^{1}H-^{15}N-HSQC (800 MHz/81.05 MHz, d6-DMSO) δ_H/δ_N in ppm 8.60/117.8 (HN-amide).^{31}P (242.94 MHz, d_6-DMSO) δ_P in ppm -1.75 ($(RO)_3P=O$)

G2-PPI-PC (4): Compound 2 (1.5 eq, 50 mg, 0.12 mmol) and TFFH (1.7 eq, 39 mg, 0.15 mmol) were dissolved in NMP (1 mL) and DIPEA (3 eq., 25 µL) was added to the solution and mixed for 15 mins for intermediate activation. PPI-G2 (1 eq., 8.4 mg, 11 µmol, end-group conc.: 0.086 mmol) was added and the reaction was stirred for 20 h at room temperature. Work-up was carried out as for compound **3**. Yield: 31.3 mg (75.3%). SEC: One peak, t_R 10.4 min. FT-IR (ATR) ν in cm^{-1} 792 ($(RO)_3P=O$), 1063/1215 (C-O, ester), 1645 (C=O, amide), 1726 (C=O, ester). ^{1}H-^{13}C-HSQC NMR (800 MHz/200 MHz, d_6-DMSO) δ_H/δ_C in ppm 1.16/16.9 (CH3), 1.80/20.7 (CH_2), 1.80/23.9 (CH_2), 2.41/36.0 (CH_2-C=O), 2.69/39.0 (CH-C=O), 2.71/27.9 (CH_2-S), 2.79/2.62/35.0 (CH_2-S), 2.99/50.4 (CH_2-N), 3.14/36.4 (CH_2-NH amide), 3.17/53.6 (CH_3-N^+), 3.59/65.7 (CH_2-N^+), 3.93/63.2 (CH_2-O-P), 4.13/59.2 (CH_2-O-P), 4.27/4.12/64.5 (CH_2-O-C=O). ^{1}H-^{15}N-HSQC (800MHz/81.05MHz, d6-DMSO) δ_H/δ_N in ppm 8.55/117.9 (HN-amide). ^{31}P (242.94 MHz, d_6-DMSO) δ_P in ppm -1.75 ($(RO)_3P=O$)

G3-PPI-PC (5): Compound 2 (1.5 eq, 50 mg, 0.12 mmol) and TFFH (1.7 eq, 39 mg, 0.15 mmol) were dissolved in NMP (1 mL) and DIPEA (3 eq., 25 µL) was added to the solution and mixed for 15 mins for intermediate activation. PPI-G3 (1 eq., 9.1 mg, 5.4 µmol, end-group conc.: 0.086 mmol) was added and the reaction was mixed for 20 h at room temperature. Work-up was carried out as for compound **3**. Yield: 37.3 mg (88.1%). SEC: One peak, t_R 9.8 min. FT-IR (ATR) ν in cm^{-1} 791 ($(RO)_3P=O$), 1070/1229 (C-O, ester), 1651

(C=O, amide), 1730 (C=O, ester). ^{1}H-^{13}C-HSQC NMR (800 MHz/200 MHz, d_6-DMSO) δ_H/δ_C in ppm 1.16/16.9 (CH3), 1.71/20.9 (CH_2), 1.80/24.0 (CH_2), 2.41/36.0 (CH_2-C=O), 2.69/39.9 (CH-C=O), 2.71/28.0 (CH_2-S), 2.79/2.63/35.0 (CH_2-S), 3.0/51.7 (CH_2-N), 3.10/36.3 (CH_2-NH amide), 3.17/53.6 (CH_3-N^+), 3.61/65.7 (CH_2-N^+), 3.94/63.3 (CH_2-O-P), 4.14/59.2 (CH_2-O-P), 4.26/4.15/64.5 (CH_2-O-C=O). ^{1}H-^{15}N-HSQC (800 MHz/81.05 MHz, d6-DMSO) δ_H/δ_N in ppm 8.52/118.0 (HN-amide). ^{31}P (242.94 MHz, d_6-DMSO) δ_P in ppm -1.75 ($(RO)_3P=O$)

G4-PPI-PC (6): Compound 2 (1.5 eq, 50 mg, 0.12 mmol) and TFFH (1.7 eq, 39 mg, 0.15 mmol) were dissolved in NMP (1 mL) and DIPEA (3 eq., 25 µL) was added to the solution and stirred for 15 mins for intermediate activation. PPI-G4 (1 eq., 9.5 mg, 2.7 µmol, end-group conc.: 0,086 mmol) was added and the reaction was stirred for 20 h at room temperature. Work-up was carried out as for compound **3**. Yield: 29 mg (67%). SEC: One peak, t_R 8.8 min. FT-IR (ATR) ν in cm^{-1} 791 ($(RO)_3P=O$); 1065/1217 (C-O, ester), 1651 (C=O, amide), 1732 (C=O, ester). ^{1}H-^{13}C-HSQC NMR (800 MHz/200 MHz, d_6-DMSO) δ_H/δ_C in ppm 1.16/16.9 (CH_3), 1.80/24.0 (CH_2), 2.41/36.0 (CH_2-C=O), 2.69/39.9 (CH-C=O), 2.71/27.9 (CH_2-S), 2.79/2.62/35.0 (CH_2-S), 3.00/50.4 (CH_2-N), 3.14/36.5 (CH_2-NH amide), 3.19/53.6 (CH_3-N^+), 3.58/65.7 (CH_2-N^+), 3.92/63.2 (CH_2-O-P), 4.12/59.2 (CH_2-O-P), 4.26/4.13/64.5 (CH_2-O-C=O). ^{1}H-^{15}N-HSQC (800 MHz/81.05 MHz, d6-DMSO) δ_H/δ_N in ppm 8.58/118.1 (HN-amide). ^{31}P (242.94 MHz, d_6-DMSO) δ_P in ppm -1.78 ($(RO)_3P=O$)

G5-PPI-PC (7): Compound 2 (1.5 eq, 50 mg, 0.12 mmol) and TFFH (1.7 eq, 39 mg, 0.15 mmol) were dissolved in NMP (1 mL) and DIPEA (3 eq., 25 µL) was added to the solution and stirred for 15 mins for intermediate activation. PPI-G5 (1 eq., 9.7 mg, 1.4 µmol, end-group conc.:0.089 mmol) was added and the reaction was mixed for 20 h at room temperature. Work-up was carried out as for compound **3**. Yield: 30 mg (71%). SEC: One peak, t_R 8.4 min. FT-IR (ATR) ν in cm^{-1} 791 ($(RO)_3P=O$), 1085/1217 (C-O, ester), 1651

(C=O, amide), 1732 (C=O, ester). ^{1}H-^{13}C-HSQC NMR (800MHz/200 MHz, d_6-DMSO) δ_H/δ_C in ppm 1.16/16.9 (CH_3), 1.81/23.9 (CH_2), 2.41/36.0 (CH_2-C=O), 2.70/40.0 (CH-C=O), 2.71/28.0 (CH_2-S), 2.79/2.63/35.0 (CH_2-S), 3.00/50.4 (CH_2-N), 3.14/36.4 (CH_2-N amide), 3.19/53.6 (CH_3-N^+), 3.62/65.7 (CH_2-N^+), 3.9/63.3 (CH_2-O-P), 4.12/59.2 (CH_2-O-P), 4.26/4.13/64.5 (CH_2-O-C=O). ^{1}H-^{15}N-HSQC (800 MHz/81.05 MHz, d6-DMSO) δ_H/δ_N in ppm 8.49/118.0 (HN-amide). ^{31}P (242.94 MHz, d_6-DMSO) δ_P in ppm -1.75 ($(RO)_3P=O$)

Biotin-PC (8): Compound 2 (53 mg, 0.13 mmol), biotin hydrazide (33mg, 0.13 mmol) and PyBOP (67mg, 0.13mmol) were placed in an Eppendorf tube. NMP (0.5 mL) was added, followed by N-methylmorpholine (44 uL, 0.39 mmol). The suspension was shaken overnight at r.t. gradually forming a clear solution. The mixture was added dropwise to acetone (3 mL), causing precipitation of white crystals. The suspension was centrifuged (4500 rpm, 2 min) and the supernatant was removed by decantation. The crude product was further purified by preparative HPLC, yield 15 mg (20%). HPLC: One peak, t_R 1.5-1.6 min, MS: Calcd. ($C_{24}H_{44}N_5O_9PS_2$) 641.23, found 642.31 $[M+H]^+$. FT-IR (ATR) ν in cm^{-1} 798 ($(RO)_3P=O$), 1055/1200 (C-O in ester), 1599 (C=O, hydrazide), 1681/1693 (C=O, urea), 1730 (C=O, ester), 3209 (O-H and NH stretch). ^{1}H NMR (800 MHz, d_6-DMSO) δ in ppm 1.16 (d, *J* 6.7 Hz, 3H,-CH_3), 1.30 (m, 2H, CH_2), 1.50 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 2.07 (td, *J* 7.5 Hz, 3.0 Hz, CH_2-C=O), 2.43 (m, 2H, -CH_2-C=O), 2.59 (d *J* 12.3 Hz, 1H, CH_2-S), 2.63 (dd, *J* 13.0 Hz and *J* 6.4 Hz, 1H, CH_2- S), 2.70 (m, 3H, CH_2-S, CH-C=O), 2.78 (dd, *J* 13.0 Hz and *J* 7.1Hz, 2H, -CH_2-S-), 2.82 (dd, *J* 12.3Hz and *J* 5.0 Hz, 1H, CH_2-S), 3.10 (m, 1H, CH-S), 3.14 (s, 9H, N^+-CH_3), 3.58 (m, 2H, CH_2-$N(CH_3)_3$), 3.98 (m, 2H, CH_2-O-P), 4.14-4.20 (m, 5H, CH-NH-C=O, CH2-O-C=O, R-CH_2-O-$P(O)_3$-), 4.31 (m, CH-N), 6.37 (s, 1H, NH (urea)), 6.43 (s, 1H, NH (urea)), 9.77 (d, *J* 1.3 Hz, NH (hydrazide)), 9.97 (d, *J* 1.3 Hz, NH (hydrazide)) ^{13}C NMR (200 MHz, d_6-DMSO) δ 16.5 (CH_3), 28.7 (CH_2-S), 34.6 (CH_2-S), 37.8 (CH_2), 40.4 (CH),

54.3 (CH_3-N^+), 59.8 (CH_2), 64.0 (CH_2), 64.7 (CH_2), 66.2 (CH_2-N^+), 178.1 (1C, Carbonyl: -C=O), 180.9 (1C, Carbonyl: C=O).^{1}H-^{15}N-HSQC (800 MHz/81.05 MHz, d6-DMSO) δ_H/δ_N in ppm 6.43/80.4 (HN-urea), 6.37/89.6 (HN-urea), 9.77/129.0 (HN-hydrazide), 9.96/129.7 (HN-hydrazide)

Fragmentation of rabbit anti human CRP into F(ab)$_2$: Agarose-coupled pepsin (Sigma P0609) was suspended in milliQ water at 10 mg/mL, corresponding to 300 pepsin units per mL. The suspension was washed on a glass filter with 4 volumes of 0.1 M sodium acetate pH 4.0 and then transferred to a tube in the same buffer. Rabbit anti human CRP (DAKO Q0329) was dialysed against 0.1 M sodium acetate pH 4.0 and added to the washed pepsin-agarose suspension at 1:20 (wt/wt pepsin/antibody) and the mix was incubated for 4 hours at 37 °C inverting the tube 25 times per minute. Hereafter, the reaction was stopped by adding 0.1 M Tris-HCL pH 8.8 until pH of the incubation suspension was 7.0, and the suspension was transferred to a chromatographic column and washed with 4 volumes of 0.1 M Tris-HCL pH 7.0, all of which was collected as the effluent from the column. The combined effluents were then loaded onto a protein A column equilibrated in 0.1 M Tris-HCl, pH 8.8 and incubated by 25 rpm upside down mixing for 2 hours at 37 °C, where after the run-through and four wash fractions (washing buffer: 0.1 M Tris-HCL pH 7.0) were spectrophotometrically analysed for the presence of protein. Protein containing fractions were pooled and analyzed by silver stained SDS PAGE to show the presence of F(ab)$_2$–fragments.

Immunoassays: All assays were performed in 96-well microtiter plates and all volumes were 100 µL unless otherwise indicated. All washings were done by completely filling and then emptying all wells for a total of four times. All incubations were done with slight agitation at 37 °C on a shaking table unless otherwise noted. Development of plates was done with a

tetramethylbenzidine (TMB) peroxide color substrate from Kem-En-Tec (Taastrup, Denmark), following the manufacturer's instructions, reading optical densities of wells at 450 nm subtracting unspecific coloration at 650 nm using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples were determined in duplicate. Further details are found in the immunoassay descriptions below.

CRP:PPI-PC binding assay: For the analysis of CRP binding by PC-dendrimers non-covalently immobilized on microtiter plates, Nunc Maxisorp (Thermo Scientific, no. 442404) 96-well polystyrene plates were coated overnight at 4 ^{o}C with dendrimer-PC (5 µg/mL in 0.1 M sodium carbonate pH 9.6). As negative control, underivatized PPI dendrimers were coated under the same conditions. After wash in Ca^{2+} containing washing buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.1 % Tween 20, pH 7.4) plates were blocked with washing buffer (200 µL/well) containing 5% skimmed milk powder (retail quality) for 30 minutes with agitation at room temperature (approx. 20^{o}C). This was followed by washing and then incubation for one hour with human CRP (140-11, obtained from human pleural fluid, Lee Biosolutions, USA) in washing buffer. For determination of affinity of PPI-PC:CRP binding, CRP was applied in 2-fold dilutions series from 10 ng/ml. This incubation was followed by washing and subsequently incubation with 10 µg/ml rabbit anti-hu CRP antibody (DAKO Q0329) in washing buffer for one hour. After another round of washing the final incubation was done for one hour with horse radish peroxidase (HRP) conjugated swine anti-rabbit IgG (DAKO P217) diluted 1/2000 followed by wash and development as described above. In order to show the dependence of Ca^{2+} for the binding of CRP to PC, a Ca^{2+} depleted washing buffer was prepared, replacing 10 mM $CaCl_2$ with 10 mM EDTA (ethylenediaminetetraacetic acid, sodium salt, Sigma Aldrich).

A competitive version of the above immunosassay was used for the analysis of monomeric PC (PC alone, biotinylated PC and methylester-PC-methylester, all of which were found to adsorb poorly to the microtiter plate). In the competitive assay a dilution series of the PC monomer in question (from 250 µg/ml (concentration in well)) was incubated with CRP at a fixed concentration (5 ng/ml in well) and using PPI-PC dendrimers for coating (at 5 µg/ml) (all PPI-PC generations (G1-G5) were tested, however only data for G3-PPI-PC are shown). All additional incubation steps, buffers, incubation times and development steps were done as described above.

C1q:CRP:PC and C1q:CRP:PPI-PC binding assays: For analysis of C1q binding to CRP bound to immobilized PPI-PC dendrimers a modified version of the above assay was performed in which PC-dendrimers (as well as methyl ester-PC) were coated at 1 µg/ml followed by CRP at 0.1 µg/mL and then by human C1q (HC2123, obtained from human serum, Hycult Biotech, The Netherlands) at 10 µg/mL for one hour followed by washes and incubation with mouse anti-mouse C1q (cross-reacting with human C1q)(HM 1096, Hycult Biotech, The Netherlands) at 1 µg/mL for one hour. Final incubation was with HRP-rabbit anti-mouse IgG (DAKO P260) diluted 1/2000 for one hour, before development as described above. For comparison the same assay was performed with direct coat of CRP (no PC-dendrimer coating), followed by C1q, anti C1q and HRP-anti mouse IgG as above.

Assay for C1q:CRP:PC and C1q:CRP:PPI-PC complex formation in solution: Formation of C1q:CRP:PC and C1q:CRP:PPI-PC complexes were furthermore investigated in solution. First, complexes were formed in solution in Nunc non-treated polystyrene 96-well microwell plates (Thermo Scientific, no. 269620) by incubating the PC compound in question (dendrimer or monomer) with human CRP and C1q in a total volume of 100 µL for one hour at 37 ^{o}C with slight agitation. CRP was used at 0.05 µg/mL and C1q at a starting

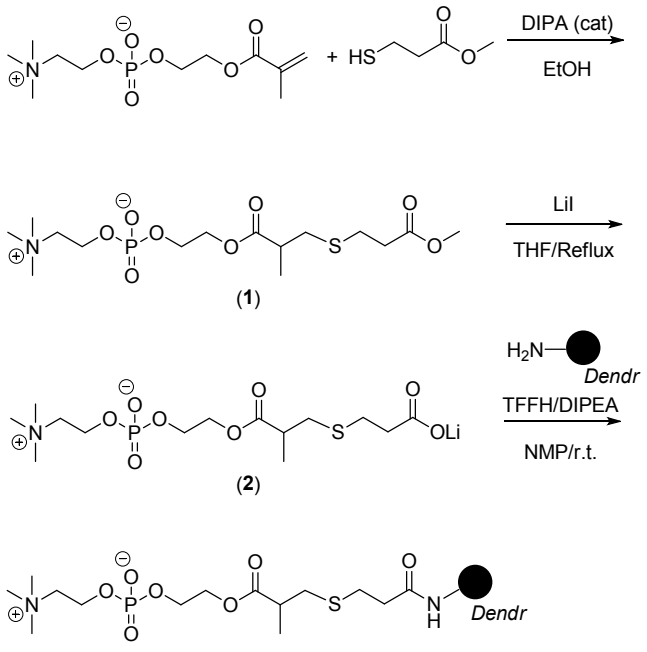
concentration of 25 µg/mL (2-fold dilution series) while the PC constructs were used at 0.25 µg/mL. Negative controls were done by leaving out CRP and PC construct, respectively. After this incubation 100 µL was transferred to a Nunc Maxisorp plate that had been pre-treated as follows: The plate was coated with $F(ab)_2$ rabbit anti-human CRP (DAKO Q0329) at 5 µg/ml in 0.1 M sodium carbonate pH 9.6 overnight at 4 ^{o}C, followed by wash, blocking and wash as above, except that blocking was done at 37 ^{o}C. The Maxisorp plate was then incubated with the C1q:CRP:PC complexes for one hour, washed and incubated with biotinylated anti-mouse C1q (Hycult Biotech, HM1096BT) 1 µg/ml in washing buffer for one hour, followed by another wash and final incubation with HRP-streptavidin (DAKO P397) at 1/2000 for one hour. Development of plates was carried out as described above.

Calculation of dissociation constants for CRP:PPI-PC binding: One binding-site approximated dissociation constants were derived from the C50% values for non-linearly fitted CRP binding curves for dilution series of CRP on solid-phase bound G1-G5 PPI-PC dendrimers using a molecular weight of 115,000 Da for the intact pentameric CRP (GraphPad Prism). For all fits R2 was above 0.99. For PC monomer binding, the same approach was used, however in a competitive immunoassay as described above, and using a molecular weight for PC of 184.15. As the monomer could not be used reproducibly for coating, the assay was performed with PPI-PC dendrimer coating, the generation of which influenced the result giving a range of K_d values, the mean of which were taken as an approximation of the real value (50 µM within a range of approx. 20-60 µM).

Results and discussion:

Chemical synthesis

Dendrimer PPI generation 1-5 with primary amine groups at their surfaces were applied as scaffolds for multimeric presentation of PC. Dendrimers modified with PC groups by other synthetic routes have been reported previously.[25, 26] However, fully derivatised dendrimer products have not been obtained. Here, we utilized the commercially available PC compound 2-methacryloyloxyethyl phosphorylcholine (MPC) to introduce PC groups onto the dendrimer surface amines. Initially, we attempted to react MPC directly with the dendrimer amines through an aza-Michael addition. However, this strategy only gave a low degree of derivatization of the dendrimer surface amines, and we instead focused on derivatizing the dendrimer amine surface groups by a more efficient amide bond formation. A carboxylic acid group was introduced to the MPC derivative by a two-step synthetic sequence. First the MPC and methyl-3-mercaptopropionate were reacted via a Michael addition (Scheme 1). The reaction takes place in ethanol at room temperature using diisopropylamine (DIPA) as the catalyst under conditions previously reported in the synthesis of similar compounds.[27]



Scheme 1. Synthesis of phosphocholine modified PPI-dendrimers. Only the chemical modification of the dendrimer surface amines to yield the PC surface groups is shown. The dendrimer scaffold displayed in Figure 2 is schematically shown as a black ball.

The thiol was used in excess as this compound (and the corresponding disulfide) could easily be separated from the polar product by extraction of these more apolar compounds into ethyl acetate. The product (**1**) was obtained in high yields (>90%) and high crude purity. The methylester group in compound (**1**) was selectively cleaved by refluxing (**1**) with LiI in THF to afford the phosphocholine compound as the lithium carboxylate (**2**). Excess LiI and small amounts of iodine formed during the reaction were removed by precipitating and washing the product in acetone. Yields were moderate to good (60-90%), with lowest yield upon scaling up this reaction to gram quantities. Finally, we coupled the PC-carboxylate (**2**) to the dendrimer surface amines by amide bond formation. Several peptide coupling agents were tested for this, including Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), 3-[Bis(dimethylamino) methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU) and Fluoro-N,N,N′,N′-tetramethylformamidinium hexafluorophosphate (TFFH). The TFFH analogue Fluoro-dipyrrolidinocarbenium hexafluorophosphate (BTFFH) was also investigated as coupling agent but resulted in the formation of dipyrrolidino-urea along with the dendrimer product (Figure 3). This urea compound was encapsulated in the dendrimer product and proved very hard to remove, even with prolonged dialysis, compared to TMU (the urea derivative formed from TFFH).

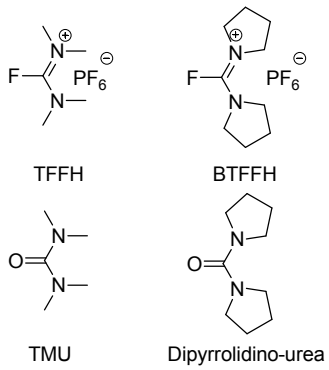
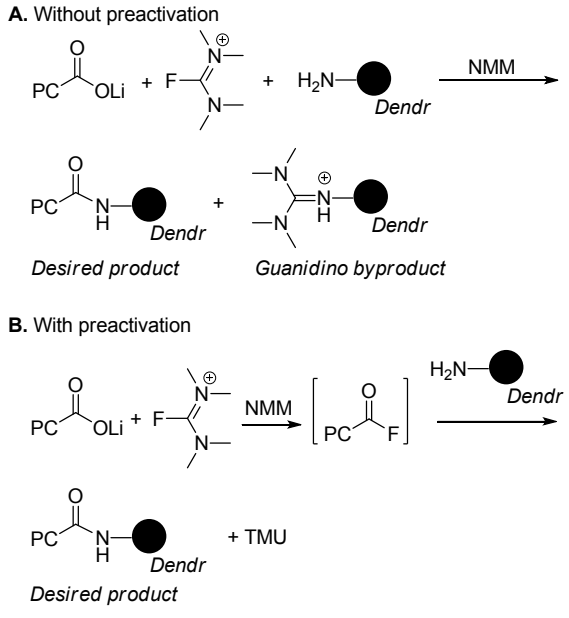


Figure 3. The structure of TFFH and BTFFH coupling agents (top) and their corresponding urea products (bottom).

TFFH was preferred due to the higher coupling efficacy found here, together with more easily removable byproducts. Here, it proved important to activate the PC-carboxylic moiety before mixing with the dendrimer, otherwise formation of a guanidine byproduct from the reaction between TFFH and the dendrimer amines was observed as a major side reaction. This side reaction was observed as an additional signal at δ 91.2 ppm in ^{1}H-^{15}N HSQC (Figure 4).



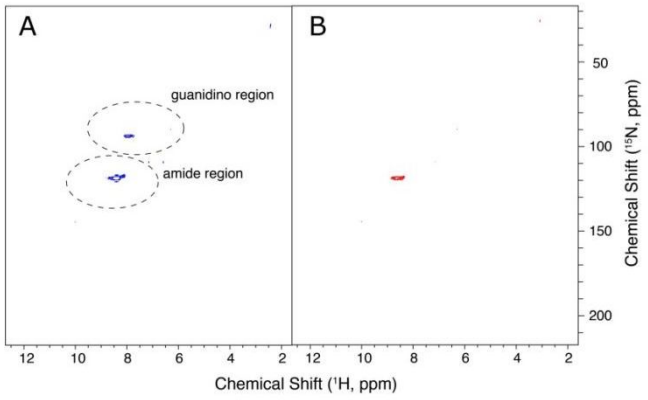


Figure 4. Top: Scheme showing the effect of preactivation on the formation of the dendrimer guanidino side product. Bottom: ^{1}H-^{15}N HSQC-NMR. Left: TFFH mediated amide bond formation between phosphocholine compound **2** and a G3 PPI dendrimer without preactivation. Right: the same TFFH mediated amide bond formation with 15 minutes of preactivation of the phosphocholine prior to the mixing with dendrimer.

The workup of the dendrimers to remove excess PC derivative **2** was initially carried out by dialysis, but this method was time consuming and only gave the dendrimer-PC products in low yields, and with some of compound **2** still present. It was found that suspending and stirring/shaking the crude dendrimer-PC products in ethanol where compound **2** has a higher solubility compared to the dendrimer-PC product, gave the dendrimer-PC compounds in good yields and high purity. However, as a consequence of higher solubility of the G1-dendrimer-PC product in ethanol, the yield of this compound was only 14%, whereas synthesis of the G2-G5 PPI-PC dendrimers gave 67-88% of the products.

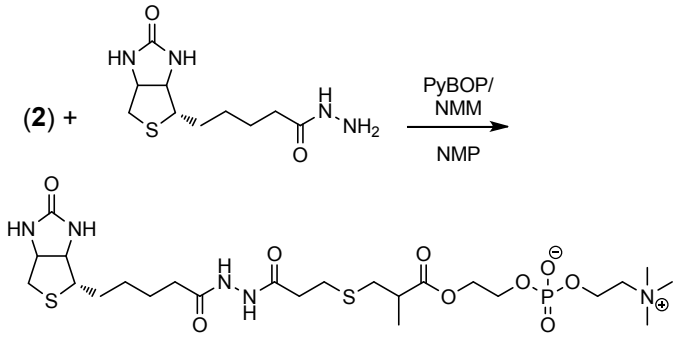
Characterization of PC decorated dendrimers by NMR

High field NMR (800 MHz) was applied to determine the structure of the dendrimer-PC products. Specifically, ^{1}H-^{15}N HSQC and ^{1}H-^{13}C HSQC were used to probe derivatization of

the primary amines at the dendrimer surface and to show that the unmodified dendrimer reactants were quantitatively transformed to the corresponding dendrimer-PC adducts during the amide bond formation. Homonuclear NOESY and TOCSY were used to confirm the proper derivatization of the dendrimer surface amines with PC through amide bond formation (See Supporting Information).

Analysis of the PPI-PC adducts by size exclusion chromatography (SEC) showed the expected decrease in retention time with increasing dendrimer generation (See Supporting Information, S11). It was observed that PC modified dendrimers formed fast eluting larger aggregates in water resulting in broad peaks with short retention time (t_R), however this could be counteracted by including 30% ethanol in the solvent leading to more defined narrow peaks with longer t_R and better correlation with the dendrimer generation number. Generally, a small peak was observed at shorter eluting time which may correspond to an adduct or complex of higher molecular weight. The retention times (t_R) of unmodified dendrimers were generally approximately 0.5 min higher than those of the corresponding PC modified dendrimer of the same generation consistent with the increased size of the PC modified dendrimers (see supporting information). Analysis by reversed phase HPLC (C_{18} modified silica) resulted in very broad and flat peaks for the PC modified dendrimers, barely distinguishable from the baseline (data not shown). This suggests that the modified dendrimers (despite their polar surface groups) are prone to hydrophobic interactions including inter-molecular binding and binding to the hydrophobic reversed phase matrix. Infrared spectroscopy confirmed the formation of amide bonds between the dendrimer and COOLi-PC (**2**) by the presence of an amide band at approximately 1650 cm^{-1}. Furthermore, IR bands show the presence of both an ester bond and phosphate ester at wavenumbers of approximately 1730 cm^{-1} and 791 cm^{-1}, respectively (see Supporting Information)

Synthesis of biotinylated PC monomer: In order to determine the binding of monovalent PC a biotin derivative of PC was synthesized via acylation of the commercially available biotin hydrazide with compound (**2**). Several acylation conditions were investigated, e.g. using uronium based coupling agents such as TFFH or HBTU. However, it was found that these coupling agents gave poor yield and higher amounts of side products. The PyBOP coupling agent was found to give the product in highest crude yield and purity. NMM was used as base as it is more hydrophilic compared to DIPEA and therefore has a higher miscibility under the reaction conditions (Scheme 2).



Scheme 2. Synthesis of biotin-PC derivative for the investigation of binding of PC to CRP.

CRP:PPI-PC and C1q:CRP:PPI-PC binding assays

A first, rough estimate of overall apparent affinity of CRP:PPI-PC complexes (G1 to G5) was obtained by a non-competitive enzyme-linked immunosorbent assays (ELISA) employing dilution series of CRP and fixed concentrations of coated (i.e. solid-phase adsorbed) PPI-PC dendrimers using 96-well polystyrene multiwell plates (see Figure 5) as well as a rabbit anti human CRP antibody for detection of CRP. In this set-up the concentration of the ligand (CRP) at half-maximal signal equals the dissociation constant (Kd) of the complex. The method has a number of shortcomings[28] and does not yield the affinity constant in solution,

as the dendrimer is immobilized. Competition-type immunoassays could potentially provide the solution phase interaction between CRP and PPI-PC. However, CRP:PPI-PC complexes were not blocked from binding to solid phase bound PPI-PC due to the polymeric nature of both ligands (data not shown). Binding affinities of monomeric PC (which did not coat well to the multiwell plates used here) were estimated by a competitive immunoassay, using monomeric PC as competitor and absorbed PC-PPI as 'catcher' of CRP. Even if the competitor molecule (monomeric PC) has a substantially different affinity from the coated 'catching' molecule (multimeric PPI-PC) an estimate of the affinity of monomeric PC towards CRP could be obtained (Figure 6). Finally, we used a combined solution phase/solid phase immunoassay to determine the functional affinity of CRP:PPI-PC complexes for C1q employing the G1-G5 PPI-PC dendrimer series as well as appropriate PC-monomer constructs (Figures 7 and 9)

CRP:PPI-PC binding assay: As described above the binding of CRP to G1-G5 PPI-PC dendrimers was investigated by detecting CRP bound to coated dendrimer by a CRP-specific antibody (Figure 5). We found that CRP bound all PPI-PC dendrimers in a titratable and saturable manner with similar maximum binding (Figure 6, top). Binding curves were approximated by a one-site specific binding fit, from which overall K_d values were derived (Table 1). In a competitive set-up, monomeric PC efficiently inhibited binding between G3-PPI-PC and CRP consistent with specificity of the interaction between the PPI-PC dendrimer and CRP, although the K_d for the PC-CRP interaction was orders of magnitude lower than for the PPI-PC dendrimers (see below), namely in the 50 μM range (Figure 6, bottom). Interestingly, both biotinylated PC (Scheme 2) and methylester-PC both showed an even weaker binding to CRP (Figure 6, bottom). Thus, modifications to the phosphate group of PC negatively affect binding of PC to CRP, which is in accordance with the model of PC-

CRP interaction, in which the binding of the phosphate group of PC to the two bound Ca^{2+}-ions on the B-surface of CRP is a major factor.[5] This dependence on Ca^{2+} is consistent with earlier work, in which it was concluded that PC in phosphodiester linkage had a significantly reduced CRP-binding compared to PC itself.[10] For the PPI-PC compounds all of the derived K_d values were in the nM range, however with subtle differences; G2 and G3 PPI-PC dendrimers displayed the strongest binding (Kd ≈ 7 nM), followed by G1 and G4 (Kd ≈ 11 nM) and with G5 as the weakest binder (Kd ≈ 20 nM). The binding was completely abolished for all PPI-PC dendrimer generations by including 10 mM EDTA in the assay buffer instead of 10 mM Ca^{2+} (not shown).

As shown in Table 1, the overall affinity for CRP increased almost proportionally with the number of head groups from G1-PPI-PC to G2-PPI-PC suggesting an additive effect of increasing dendrimer PC valency from four to eight. However, increasing the number to 16 (G3) and 32 (G4) does not follow this trend, and for G5 an antagonistic effect can be seen. Per PC group the affinity increase compared to the monomer gradually declines from 1000-fold increase for the G1-PPI-PC compound to 40-fold for G5. The findings are consistent with multivalent (valency at or above 4) binding of the dendrimers increasing affinity, while higher generations show declining affinity, possibly due to steric crowding

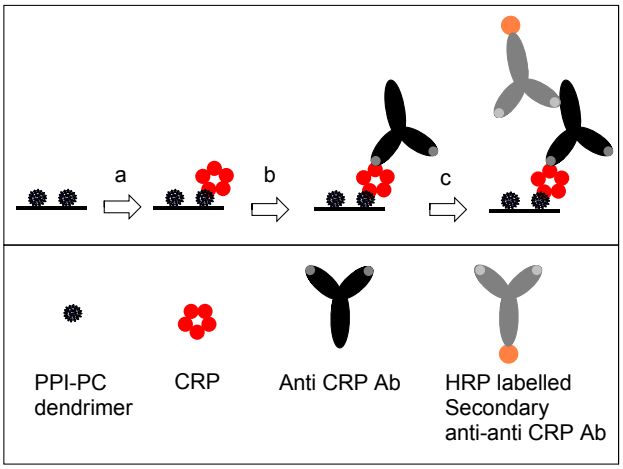
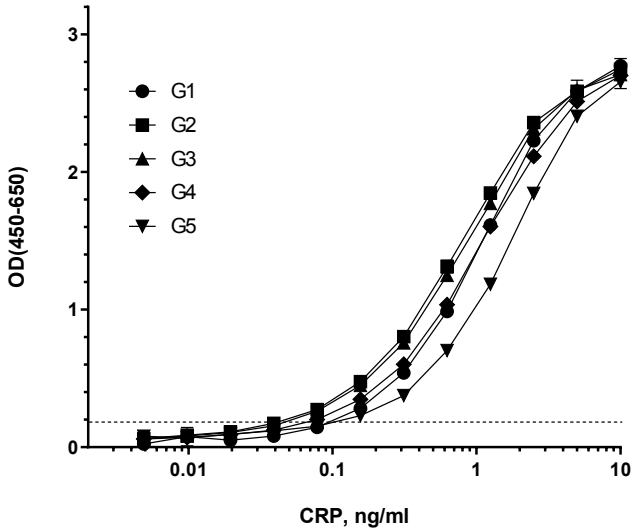


Figure 5. CRP:PPI-PC binding solid phase assay (ELISA) setup, a) Binding of CRP to a well with adsorbed PPI-PC compound, b) Binding of primary anti-CRP antibody, c) Binding of HRP (horse radish peroxidase)-labelled secondary antibody. In the competitive set-up used for PC monomers, a dilution series of the PC monomer was included in step a. Molecules are approximately drawn to scale.



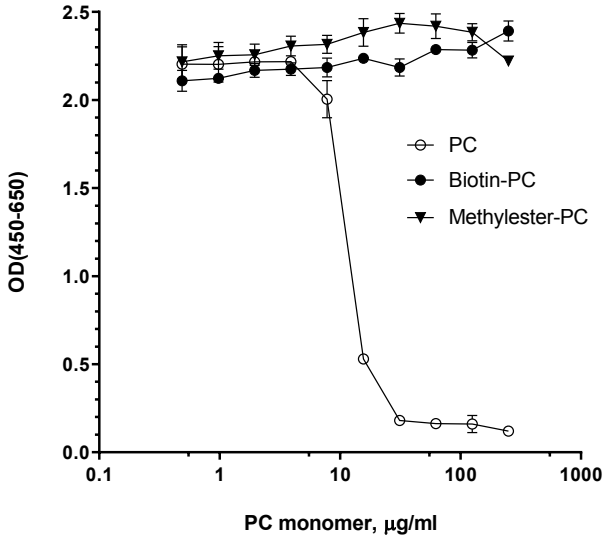


Figure 6. Top: Titration curves of CRP on solid phase bound G1 to G5 PPI-PC dendrimers (double determinations, +/- SD). Background OD (no dendrimer) was below 0.01. Dotted line indicates mean signal obtained with underivatized G1 to G5 PPI dendrimers (no PC groups, mean and SEM, 0.172 +/-0.005) in the

coating layer. Bottom: Competitive immunoassay using PC monomers (PC, biotin-PC, methylester-PC, as indicated) as inhibitors, keeping the CRP concentration constant, and using G3-PPI-PC for coating (see text for description); double determinations, +/- SD.

Table 1. Overall dissociation constants (K_d) of CRP:PPI-PC complexes derived from binding curves of dilution series of CRP on solid-phase bound PC-dendrimers 1-5 (Figure 6, top) assuming one-site specific binding (EC50 = K_d). K_d of PC monomer was determined in a competitive assay as described (IC50 = K_d, Figure 6, bottom). Affinity increases are taken relative to the PC monomer.

Compound (n_{PC})	**K_d, nM**	**Approx. affinity increase**	**Approx. affinity increase/PC group**
PC (1)	50000	1	1
G1-PPI-PC (4)	11.91	4201	1050
G2-PPI-PC (8)	7.15	6993	874
G3-PPI-PC (16)	8.04	6219	389
G4-PPI-PC (32)	10.64	4699	147
G5-PPI-PC (64)	20.78	2406	38

C1q:CRP:PPI-PC binding assay: Multiwell plate-adsorbed G1-G5 PPI-PC dendrimers were screened for their ability to promote binding of C1q to the CRP (see Figure 7 for the ELISA set-up and Figure 8 for the results of the assay) at a constant concentration of CRP (0.1 µg/ml) and employing all dendrimers at 1 µg/ml. All PPI-PC dendrimer generations promoted

binding of C1q both compared with directly coated CRP and with CRP exposed to a methylester-PC monomer. The highest gain in C1q binding was observed with G2- and G3-PPI-PC dendrimers, followed by G4 and G5, with G1 having the smallest effect. CRP alone gave an OD at around 0.45 (Figure 8, dotted line 'no PC-construct'), clearly lower than the OD values obtained for all PPI-PC-dendrimers, but higher than observed with the methylester-PC monomer. This suggests that methylester-PC monomer coating decreased the number of CRP molecules available for C1q binding to below that being available by the direct coating of CRP with no discernible compensatory effect on CRP's ability to bind C1q. Taking the negligible CRP-binding of methylester-PC described above into account (Figure 6), it is not surprising that the methylester-PC did not induce CRP to bind C1q. It should be noted that the resulting OD in this assay is both a function of the amount of CRP being bound by the coating PPI-PC dendrimer (which is at least partly related to the coating ability of the PPI-PC dendrimer in question) as well as a function of the influence of the PPI-PC dendrimer binding on the C1q binding of CRP. Also, a non-negligible direct binding between C1q and coated PPI-PC dendrimer was observed in controls omitting CRP (around OD=0.6, only tested for G3, not shown). It was therefore not attempted to further modify this assay to estimate C1q affinities for the different CRP:PPI-PC complexes.

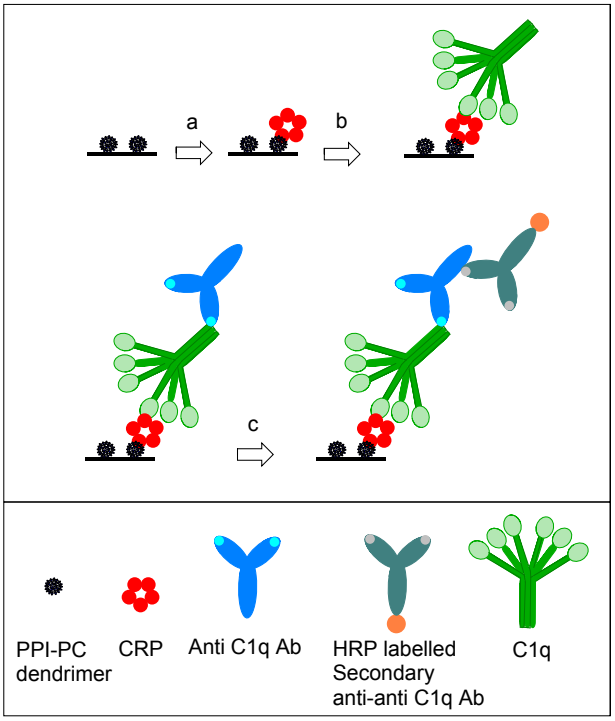


Figure 7. C1q:CRP:PPI-PC solid phase binding assay a) Binding of CRP to a well with adsorbed PPI-PC dendrimers forming solid-phase bound CRP:PPI-PC complexes, b) Binding of C1q to CRP in the CRP:PPI-PC complex and detection by anti-C1q, c) Binding of HRP-labelled anti-IgG antibody to the first antibody. Molecules are approximately drawn to scale.

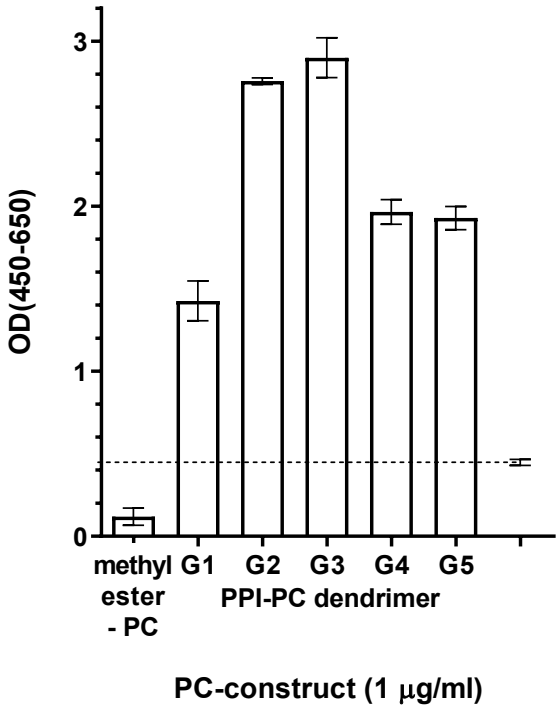


Figure 8. Effect of PPI-PC generation on binding of C1q to CRP in the C1q binding assay (as described in Figure 7). All dendrimers were coated at 1 μg/ml. Controls included coating with monomeric methylester-PC and performing the assay without any PC-construct (direct coating of CRP, dotted line), respectively. C1q was used at 10 μg/mL. Mean of double determinations, +/- SD are indicated.

Assay for C1q binding to CRP:PPI-PC complexes in solution: Due to the inability of the solid phase assay described above to separate effects of PPI-PC 'coatability' from the ability of the CRP:PPI-PC complex to bind C1q, the formation of ternary C1q:CRP:PPI-PC complexes was instead allowed to take place in solution and subsequently analyzed in a solid phase assay, sandwiching the full complex between a capture and a detection antibody (Figure 9). This solution phase approach also allowed for straightforward testing of the effect of weakly coating PC monomers on the ability of CRP to bind C1q. For the analysis of solution-phase formed C1q:CRP:PPI-PC the set-up depicted in Figure 9 was used; here the

ternary complex was immobilized through CRP by solid-phase coated anti CRP antibody, and the C1q component of the complex was detected by an anti-C1q antibody. By titrating C1q in solution binding curves allowing the determination of Kd for the binding of C1q to the CRP part of the CRP:PPI-PC complex could be obtained. As expected we observed that C1q bound directly to the Fc part of the anti CRP antibody used for catching CRP (not shown). Therefore the antibody was subjected to enzymatic fragmentation and only its $F(ab)_2$-fragment was used in the assay. This resulted in no detectable unspecific binding of C1q in the absence of CRP (not shown). The $F(ab)_2$ anti CRP antibody also did not interfere to any appreciable degree with either PPI-PC dendrimer or C1q binding by CRP (not shown). The assay confirmed the finding shown in Figure 8 that binding of C1q to CRP was dependent on CRP interacting with PPI-PC, as leaving out the PPI-PC dendrimer from the incubation cocktail abolished binding of C1q (Figure 10). By titrating C1q, relative affinities of C1q for the CRP:PPI-PC complex as a function of dendrimer generation and PC-construct type were determined by the sandwich ELISA (Figure 9). First, it could be observed that the difference between dendrimer generations in the binding of C1q to CRP:PPI-PC complexes was much bigger than observed for binding of CRP to the different PPI-PC generations (compare with Figure 6 and Table 1); C1q binding with CRP:PPI-PC complexes incorporating G2, G3, and G4-PPI-PC were similar, while the CRP:PPI-PC (G5) complex displayed a significantly decreased C1q binding and the G1 CRP:PPI-PC complex bound even more weakly, close to the binding seen for PC monomer-complexed CRP and non-dendrimer bound CRP. In summary, the following trend for relative C1q binding affinities was found for CRP:PPI-PC and CRP:PC-monomer complexes: G3 ≈ G2 ≈ G4 > G5 > G1 ≈ PC monomer. Second, although the binding curves are incomplete and indicative of a complex binding behavior, the affinity of C1q for CRP:PPI-PC complexes for the best

binding dendrimers (G2, G3, and G4) K_d values can be estimated to be around 50 nM, assuming a molecular weight of 410 kDa for the human C1q hexamer and setting the C1q concentration at half maximum signal to around 20 µg/ml (Figure 11).

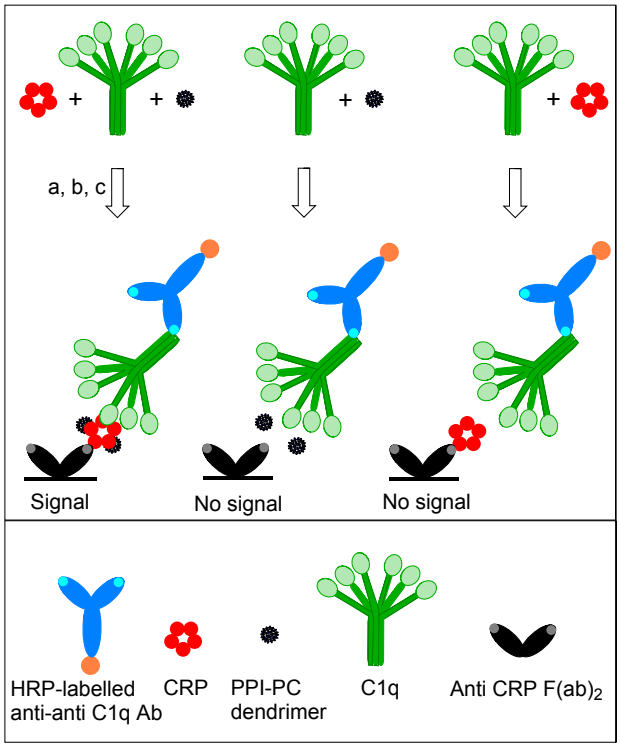


Figure 9. Combined solution and solid phase assay for C1q to CRP binding upon binding between CRP and PPI-PC dendrimers. a) Formation of C1q:CRP:PPI-PC complex by mixing of CRP with PPI-PC and subsequent addition of C1q in solution. b) Capture of C1q:CRP:PPI-PC complex by solid-phase adsorbed anti CRP $F(ab)_2$, c) detection of bound C1q by HRP labelled secondary anti-anti C1q antibody. (Molecules are approximately drawn to scale).

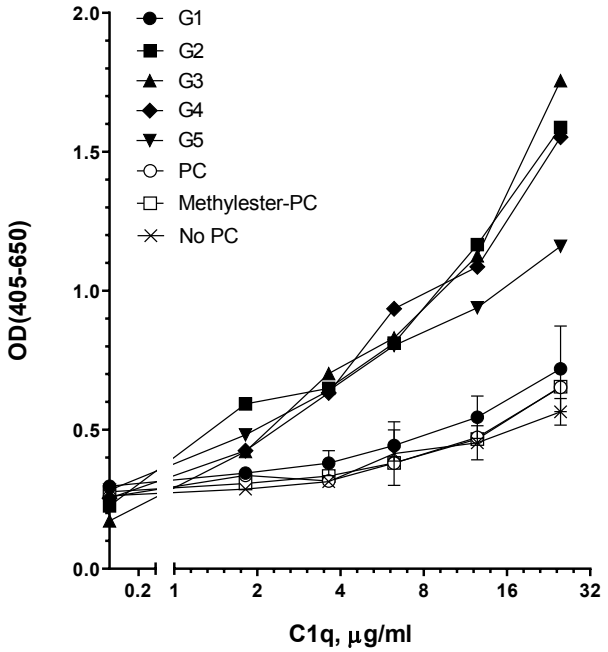


Figure 10. Titration curves of C1q in solution phase assay (see text and Figure 9) showing binding of C1q to CRP:PPI-PC dendrimer complexes with different generations of dendrimers. Controls include methylester-PC (compound 1) and underivatized PC as well as running the assay in the absence of any PC-construct ('No PC'). Means of double determinations +/- SD are shown.

Conclusion

We prepared phosphocholine (PC) decorated PPI dendrimers useful for emulating the binding of pentavalent CRP to multimeric PC clusters occurring in disrupted/compromised cell membrane lipid bilayers. The binding of these PPI-PC dendrimers to CRP and the binding of the resulting complexes to complement factor C1q were analyzed using purified human CRP and customized immunoassays. In contrast to earlier studies employing molecularly undefined multiple PC presentation at surfaces or in liposomes, we utilize nano-scaled, spherical PPI dendrimers as scaffolds for presenting PC in defined numbers and geometry to CRP. This approach allowed the straightforward investigation of the affinity of

CRP for different generations of molecularly defined PPI-PC dendrimers displaying 4, 8, 16, 32 and 64 surface-localized PC groups respectively.

The PC-decorated PPI dendrimers were successfully synthesized in satisfactory yields by a three step synthetic sequence employing chemoselective chemistry and using a TFFH preactivation protocol to bind PC to dendrimer surface amino groups. The use of TFFH allowed easier product workup compared to other coupling agents. NMR techniques proved the dendrimers to be fully modified.

We demonstrate that CRP binds to PC (the phosphate monoester) with micromolar affinity while PC phosphodiester compounds do not bind to CRP. However, when PC is presented on G1-G5 PPI dendrimers in its phosphodiester form, a more than 1000-fold increase in affinity compared to the PC monoester is observed, - an increase clearly beyond possible additive effects. There are slight variations between dendrimer generations as G2 (8 PC surface groups) and G3 (16 PC surface groups) PPI dendrimers bind most strongly, closely followed by G1 (4 PC surface groups), G4 (32 PC surface groups), and G5 (64 PC surface groups). Although it is not known at this point to which extent this dramatic increase in affinity for CRP of the PPI-PC compared to PC monoester is due to a dendritic effect or not, this seems highly likely. Geometric constraints may favor the binding of less crowded G2 and G3 PPI-PC dendrimers compared to G4 and G5 with their more densely packed surfaces.

Additionally, C1q binding by CRP is found to depend critically on multimeric PC-dendrimer mediated activation of CRP. Binding of G2, G3 and G4 PPI-PC resulted in similar affinities of CRP for C1q, while G5 was somewhat less efficient and G1 did not activate CRP much above that achieved by the PC monomer, both being quite close to the background of the assay, i.e. using non-activated CRP.

Thus, dendrimer based PC multimers can be used to reproduce central features of CRP binding in biological systems, including the dramatically increased binding affinity to multimeric PC and the subsequent promoting effect on C1q binding. This molecular system also reproduces the peculiar finding that un-clustered PC diesters bind CRP very poorly while PC diesters in multimeric clusters, such as lyso-phosphatidylcholine in biological membranes and dendrimer-supported phosphocholine in this study, bind CRP with high affinity. This invites a closer study of the relation between PC-phosphodiester cluster geometry – which can be varied in a defined and systematic way using the dendrimer approach – and CRP-binding and -activation. To get a more complete picture of binding strengths and geometries, alternative methods such as surface plasmon resonance analysis and isothermal titration calorimetry in combination with molecular dynamics simulations will have to be investigated. The presented dendrimer platform for preparing well-defined PC oligomer bundles lends itself to expanding such investigations to novel geometries. Finally, synthetic PC clusters such as dendrimer based PC multimers may have a big potential in their own right as drugs modifying the innate immune activity of CRP.

Acknowledgements

Henriette Vorsholt is gratefully acknowledged for her excellent laboratory assistance in performing affinity assays. Jonas Jørgensen is gratefully acknowledged for his support in the synthesis of compound 1. High-field NMR measurements were conducted at the DTU NMR Center, supported by the Villum Foundation.

Supporting information

Compound (1), analytical data, (^{1}H-NMR, ^{13}C-NMR, H,H-COSY, FT-IR, HPLC-MS, SEC-HPLC). Compound (2), analytical data, (^{1}H-NMR, ^{13}C-NMR, H,H-COSY, FT-IR, HPLC-MS, SEC-HPLC). General approach for NMR analysis of PPI-PC products. ^{1}H, ^{13}C-HSQC for PPI-PC products, generation 1-5 (compound 3-7). ^{1}H-^{13}C-HSQC assigned spectrum of native (uncoupled) PPI-dendrimer atom positions, as shown for a generation 2 dendrimer. PPI/PPI-PC ^{1}H, ^{13}C-HSQC overlay (G2-G4). ^{31}P NMR spectra of PPI-PC products (G1-G5). ^{1}H-^{31}P HSQC spectra of coupled PPI-PC dendrimers (G1-G5). ^{1}H-^{13}C-HSQC, comparison of the product from either BTFFH or TFFH mediated coupling reaction (G3). FT-IR, PPI-PC dendrimers (G1-G5). Size exclusion chromatotography (SEC-HPLC) at 220 nm of PPI-PC dendrimers (G1-G5). Biotin-PC, Compound (8), analytical data (^{1}H-NMR, ^{1}H, ^{13}C-HSQC, ^{1}H, ^{15}N-HSQC, FT-IR, HPLC-MS)

References

(1) Pepys, M.B.; Hirschfield, G.M. C-reactive protein: a critical update, *J. Clin. Invest*. **2003**, *111* (12), 1805-1812.

(2) Rogowski, O.; Shapira, I.; Toker, S.; Melamed, S.; Shirom, A.; Zeltserm, D.; Berliner. S. Very low C-reactive protein in apparently healthy individuals: physiological status or just a reflection of an improved health profile. *Biomarkers* **2007**, *12* (6), 645-656

(3) Zacho, J.; Tybjærg-Hansen, A.; Jensen, J. S.; Grande, P.; Sillesen, H.; Nordestgaard, B. G. Genetically elevated C-reactive protein and ischemic vascular disease. *New England J. Med.* **2008**, *359* (18), 1897-1908

(4) Casas, J.P.; Shah, T.; Hingorani, A.D.; Danesh, J.; Pepys, M.B. C-reactive protein and corony heart disease: A critical review. *J. Intern. Med.* **2008**, *264* (4), 295-314

(5) Thompson, D.; Pepys, M.B.; Wood, S.P. The physiological structure of human C-reactive protein and its complex with phosphocholine, *Structure* **1999,** *7*, 169-177.

(6) Young, N.M.; Footea, S.J.; Wakarchukb, W.W., Review of phosphocholine substituents on bacterial pathogen glycans: synthesis, structures and interactions with host proteins, *Mol. Immunol*. **2013**, *56* (4), 563– 573

(7) Pepys, M.B.; Baltz, M.L. Acute Phase Proteins with Special Reference to C-Reactive Protein and Related Proteins (Pentaxins) and Serum Amyloid A Protein, *Adv. Immunol*. **1983**, *34*, 141-212.

(8) Christopeit, T.; Gossas, T.; Danielson, U.H. Characterization of Ca2+ and phosphocholine interactions with C-reactive protein using a surface plasmon resonance biosensor, *Anal. Biochem*. **2009**, *391* (1), 39-44.

(9) Volanakis, J.E. Human C-reactive protein: expression, structure, and function, *Mol. Immunol*. **2001**, *38* (2-3), 189-197.

(10) Gotschlich, E.; Liu, T.-Y.; Oliveira, E. Binding of C-reactive protein to C-carbohydrate and PC-substituted protein, *Ann. N.Y. Acad. Sci., USA* **1982**, *389*, 163-171.

(11) Volanakis J.E.; Wirtz, K.W.A. Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. *Nature* **1979**, *281* (5727), 155-157.

(12) Kurosawa, S.; Nakamura, M.; Park, J.-W.; Aizawa, H.; Yamada, K.; Hirata, M. Evaluation of a high-affinity QCM immunosensor using antibody fragmentation and 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer. *Biosens. Bioelectr*. **2004**, *20* (6), 1134-1139.

(13) Marnell, L.; Mold, C.; du Clos, T.W. C-reactive protein: Ligands, receptors and role in inflammation, *Clin. Immunol.* **2005**, *117* (2), 104-111.

(14) Kaplan, M.H.; Volanakis, J.E. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. *J. Immunol.* **1974**, *112* (6), 2135-2147.

(15) Siegel, J.; Osmand, A.P.; Wilson, M.F.; Gewurz, H. Interactions of C-reactive protein with the complement system. II. C-reactive protein-mediated consumption of complement by poly-L-lysine polymers and other polycations. *J. Exp. Med.* **1975,** *142* (3), 709-721.

(16) Shrive, A.K.; Cheetham, G.M.T.; Holden, D.; Myles, D.A.; Turnell, W.G.; Volanakis, J.E.; Pepys, M.B.; Bloomer, A.C.; Greenhough, T.J. Three dimensional structure of human C-reactive protein. *Nat. Struct. Biol.* **1996**, *3* (4), 346-354.

(17) Pepys, M.B.; Hirschfield, G.M.; Tennent, G.A.; Gallimore, J.R.; Kahan, M.C.; Bellotti, V.; Hawkins, P.N.; Myers, R.M.; Smith, M.D.; Polara, A.; Cobb, A.J.; Ley, S.V.,;Aquilina, J.A.; Robinson, C.V.; Sharif, I.; Gray, G.A.; Sabin, C.A.; Jenvey M.C.; Kolstoe, S.E.; Thompson, D.; Wood, S.P. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature* **2006**, *440* (7088), 1217-1221.

(18) Tomalia, D. A.; Christensen, J. B.; Boas, U. *Dendrimers, dendrons and dendritic polymers*. Cambridge University Press 2012

(19) For an early review focused on biological uses of dendrimers, see e.g. Boas, U.; Heegaard, P. M. H. Dendrimers in drug research. *Chem. Soc. Rev.* **2004**, *33* (1), 43-63.

(20) Pagé, D; Roy, R. Synthesis and biological properties of mannosylated starburst poly(amidoamine) dendrimers. *Bioconjugate Chem.* **1997**, *8* (5), 714-723.

(21) Lundquist, J. J.; Debenham, S. D.; Toone, E. J. Multivalency Effects in Protein−Carbohydrate Interaction: The Binding of the Shiga-like Toxin 1 Binding Subunit to Multivalent C-Linked Glycopeptides. *J. Org. Chem.* **2000**, *65* (24), 8245-8250.

(22) Nishikawa, K.; Matsuoka, K.; Watanabe, M.; Igai, K.; Hino, K.; Hatano, K.; Yamada, A.; Abe, N.; Terunuma, D.; Kuzuhara, H.; Natori Y. Identification of the optimal structure required for a Shiga toxin neutralizer with oriented carbohydrates to function in the circulation. *J. Infect. Dis.* **2005**, *191* (12), 2097-2105.

(23) Nagahori, N.; Lee, R. T.; Nishimura, S.-I.; Pagé, D.; Roy, R.; Lee, Y. C. Inhibition of adhesion of type 1 fimbriated Escherichia coli to highly mannosylated ligands. *Chembiochem* **2002**, *3* (9), 836-844.

(24) Sorensen, N. S.; Boas, U.; Heegaard, P. M. H. Enhancement of Muramyldipeptide (MDP) Immunostimulatory Activity by Controlled Multimerization on Dendrimers. *Macromol. Biosci.* **2011**, *11* (11), 1484–1490.

(25) Heegaard, P. M. H.; Pedersen, H. G.; Jensen, A. L.; Boas, U. A robust quantitative solid phase immunoassay for the acute phase protein C-reactive protein (CRP) based on cytidine 5'-diphosphocholine coupled dendrimers. *J. Immunol. Meth.* **2009**, *343* (2), 112-118.

(26) Svenningsen, S., W.; Janaszewska, A.; Ficker, M.; Petersen, J. F.; Klajnert-Maculewicz, B.; Christensen, J. B. Two for the price of one: PAMAM-dendrimers with mixed phosporyl choline and oligomeric poly(caprolactone) surfaces. *Bioconjugate Chem.* **2016**, *27* (6), 1547-1557.

(27) Matsuno, R.; Takami, K.; Ishihara, K. Simple Synthesis of a Library of Zwitterionic Surfactants via Michael-Type Addition of Methacrylate and Alkane Thiol Compounds. *Langmuir* **2010**, *26* (16), 13028-13032.

(28) Goldberg; M. E.; Djavadi-Ohaniance, L. Methods for measurement of antibody/antigen affinity based on ELISA and RIA. *Curr. Opin. Immunol.* **1993**, *5* (2), 278-281.

Table of Contents/Graphical abstract:

Phosphocholine-Decorated PPI-Dendrimers Mimic Cell Membrane Phosphocholine Clusters and Tune the Innate Immune Activity of C-Reactive Protein

Ulrik Boas, Mohammad Mahdi Daoud, Sebastian Meier, Tobias Hegelund Olsen, Kristoffer Gehring, Ditte Juhl Mogensen, Peter M. H. Heegaard