Nanoparticle Targeting

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Polyethylene Glycol Backfilling Mitigates the Negative Impact of the Protein Corona on Nanoparticle Cell Targeting**

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Abstract: *In protein-rich environments such as the blood, the formation of a protein corona on receptor-targeting nanoparticles prevents target recognition. As a result, the ability of targeted nanoparticles to selectively bind to diseased cells is drastically inhibited. Backfilling the surface of a targeted nanoparticle with polyethylene glycol (PEG) molecules is demonstrated to reduce the formation of the protein corona and re-establishes specific binding. The length of the backfilled PEG molecules must be less than the length of the ligand linker; otherwise, PEG interferes with the binding of the targeting ligand to its corresponding cellular receptor.*

Nanoparticles are being developed for the diagnosis and treatment of a variety of diseases.[1] The surfaces of these nanoparticles are typically functionalized with antibodies,[2] peptides,[3] or aptamers[4] that enable them to recognize specific receptors on cell surfaces. This allows the nanoparticles to selectively accumulate in diseased tissues, which could enhance the diagnostic sensitivity or therapeutic index while preventing accumulation in off-target tissues. In order to improve the efficiency of nanoparticle delivery into diseased tissues, researchers have focused on understanding how targeted nanoparticles interact with biological components such as blood,[5] tissues,[6] and cells.[7] An emerging body of literature demonstrates that when nanoparticles are introduced into the blood, serum proteins interact with the nanoparticle surface to form a protein corona.[8] These adsorbed proteins affect nanoparticle pathophysiology[9] and mask the ability of targeted nanoparticles to interact with cell receptors, thereby leading to a loss of targeting specificity.[10] To suppress protein adsorption, researchers often functionalize the nanoparticle surface with the antifouling polymer polyethylene glycol (PEG).[11] In order to properly design target-specific nanoparticles, a detailed understanding of the amount of PEG to apply and type of PEG to use is essential to effectively reduce serum-protein–nanoparticle interactions. To date, it is unclear whether PEG backfilling can improve the specific binding of nanoparticles to a targeted cell population by overcoming the inhibitory effect of the protein corona. In this study, we investigated whether backfilling receptor-targeting nanoparticles with PEG could suppress serum protein adsorption and re-establish binding specificity.

50 nm gold nanoparticles functionalized with fluorescent Herceptin were synthesized for the purpose of this study (Figure 1 A, Table S1 in the Supporting Information). We used gold nanoparticles as a model system because of their ease of synthesis and[12] characterization,[13] and their relevance in targeted delivery.[14] Herceptin was chosen as the biorecognition molecule because of its prevalent use for the therapeutic and diagnostic targeting of the ErbB2 receptors in breast cancer.[15] Amines on the Herceptin molecule were labeled with the near-infrared-emitting dye Alexa Fluor 647 by using amine-reactive succinimidyl ester chemistry. The high quantum yield and near-infrared emission of the Alexa Fluor dye minimizes gold surface quenching[16], thus making this fluorophore suitable for use as a fluorescent reporter for the nanoparticle. UV/Vis spectrophotometric measurements confirmed that an average of 1.8 dye molecules were conjugated per Herceptin molecule. The low dye-to-protein ratio allows other primary amine groups on Herceptin to be further chemically modified with a 5 kDa orthopyridyldisulfide polyethyleneglycol *N*-hydroxysuccinimide ester (OPSS-PEG-NHS ester, Figure 1 A). Previously, we found that this extra step in the nanoparticle-coating process increased the strength of ligand binding to the surface of the gold nanoparticle (unpublished data). A similar strategy was used by Chattopadhyay et al. to successfully conjugate Herceptin to gold nanoparticles.[17] To gauge the range in which the conjugated Herceptin retains binding specificity for the ErbB2 receptors, we performed cell-binding experiments with serially diluted OPSS-PEG-NHS ester conjugated to Herceptin. Upon treatment of ErbB2+ SKBR3 cells with these Herceptin conjugates, we found that the specific binding of Herceptin is preserved when there are fewer than 125 OPSS-PEG-NHS ester molecules added per Herceptin molecule (Figures S1, S2 in the Supporting Information). Finally, the thiolated and fluorescently labeled Herceptin was incubated with gold nanoparticles at a molar ratio of 21:1, and approximately 16.5 Herceptin molecules were conjugated per nanoparticle (Table S1). To stabilize the nanoparticles and prevent protein adsorption, additional methoxy-terminated polythethyleneglycol (mPEG) molecules were added at 6 PEG/nm^2 to fully saturate or "backfill" the remaining gold nanoparticle surface (Figure 1 A). The hydrodynamic size and surface charge of all of the nanoparticle designs was characterized (Table S1).

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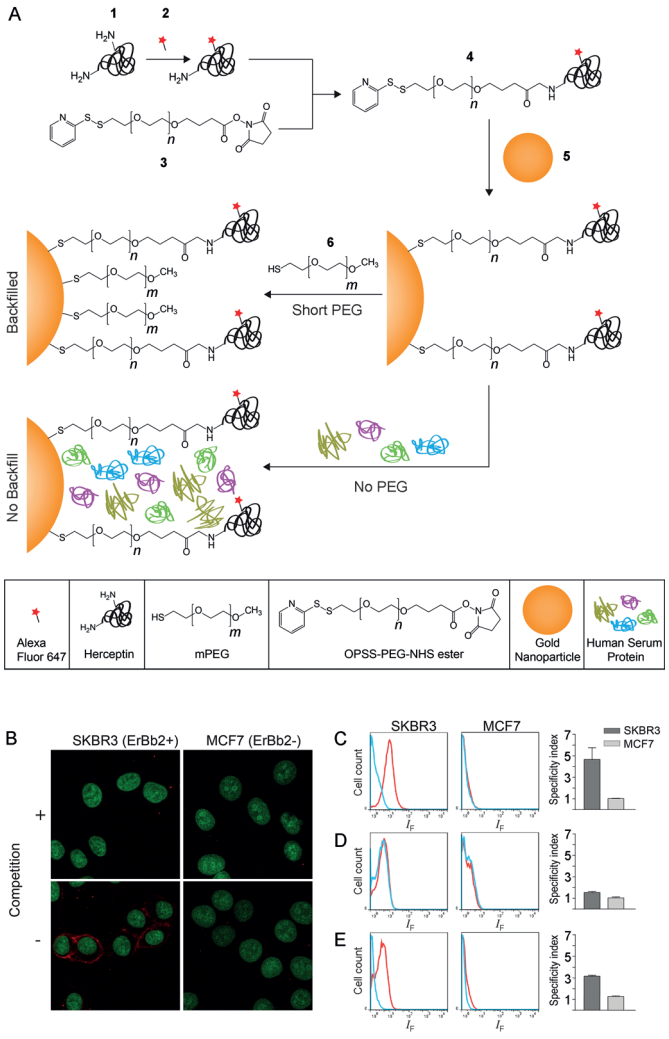

Figure 1. Binding specificity is reduced by serum-protein adsorption but this effect is alleviated by PEG backfilling. A) Herceptin (**1**) was initially conjugated to Alexa Fluor 647 (AF647; **2**) through amine-reactive succinimidyl ester chemistry, followed by a second conjugation step with orthopyridyldisulfide polyethyleneglycol *N*-hydroxysuccinimide ester (OPSS-PEG-NHS ester; **3**) to generate OPSS-PEG-Herceptin-AF647 (**4**). The surfaces of the 50 nm gold nanoparticles (**5**) were functionalized with (**4**) and backfilled with methoxy-polyethylene glycol (mPEG, **6**) through thiol coordination. When the nanoparticles were exposed to human serum, there was little binding of the serum proteins to the nanoparticles with backfilling, whereas without backfilling, there was substantial binding. The molecular weight of the PEG groups are denoted by n and m, where $n = 5$ kDa, and $m = 1$, 2, 5, or 10 kDa. B) Herceptin-conjugated nanoparticles were incubated with SKBR3 or MCF7 cells in addition to a 50-fold molar excess of unlabeled Herceptin (competition) to assess the specificity of ErbB2 receptor binding. The red fluorescence shows binding to the cell surface and the nuclei are stained green. C) The ErbB2-receptor binding specificities of these nanoparticles were quantified through flow cytometry. Red and blue lines represents cells incubated in the absence and presence of competitive Herceptin molecules, respectively. Herceptin-conjugated nanoparticles without (D) and with (E) PEG were exposed to serum and subsequently incubated with SKBR3 and MCF7 cells. Error bars represent the standard error, where $n = 3$. I_F = fluorescence intensity, cell count = normalized number (all events included) of cells counted.

We characterized the ErbB2 receptor expression on ErbB2+ SKBR3 and ErbB2− MCF7 cells by introducing an excess of unlabeled Herceptin to compete with labeled Herceptin for the ErbB2 receptors. We found that a 50-fold excess of unlabeled Herceptin was sufficient to effectively block the binding of the conjugated Herceptin (Figure S3). Next, we tested the binding specificity of the targeted nanoparticles to the SKBR3 cell line. The confocal image showed specific nanoparticle interaction with the membrane of the SKBR3 cells in the absence of competing ligands (Figure 1 B). The nanoparticles did not interact with the SKBR3 or MCF7 cells when the ErbB2 receptors were competitively bound by unlabeled Herceptin molecules (Figure 1 B). Flow cytometry was also used to quantitatively determine the binding specificity of the nanoparticles. As a normalization procedure, the specificity index for nanoparticle–cell interaction was calculated as follows [Eq. (1)]:

$$\text{Specificity index} = \frac{A}{B} \quad (1)$$

Where A and B refer to the fluorescence signals resulting from binding of the nanoparticle bioconjugates to cells in the absence (A) and presence (B) of competition (i.e., addition of excess herceptin). This index acts as a numerical determinant of the ability of a nanoparticle to specifically interact with the ErbB2 receptor. Higher values indicate higher binding specificity. The Herceptin-conjugated nanoparticles have a specificity index of 4.67 and 1.02 for SKBR3 and MCF7 cells (Figure 1 C), respectively, thus showing that the nanoparticles specifically bind the ErbB2 receptor-expressing SKBR3 cells. Next, we determined whether serum-protein adsorption reduces the specificity of Herceptin-conjugated nanoparticles for the ErbB2 receptor. Herceptin-conjugated nanoparticles were treated with 100 % human serum at 10 $\mu L\,cm^{-2}$ at 37 °C (Figure 1, Figure S4) and were subsequently incubated with cells. These nanoparticles displayed visibly higher protein adsorption compared to nanoparticles not treated with human serum (Figure S5A). The nanoparticles with the adsorbed serum-protein corona were calculated to have a specificity index of 1.50 and 1.00 for SKBR3 and MCF7 cells (Figure 1 D), respectively, thus indicating a drastic reduction in binding specificity compared to the nanoparticles that were not exposed to serum. This result shows that the adsorbed serum-protein corona reduces the interaction of the Herceptin-conjugated nanoparticles with the ErbB2 receptor. A similar trend was observed when the nanoparticles were exposed to 10 % human serum (Figure S6). Furthermore, the protein-adsorbed nanoparticles did not show completely abolished total cell binding (Figure 1 D), a result that suggests that nanoparticle–cell interactions may occur through alternative pathways that are mediated by the adsorbed serum protein. Similar results were obtained by Salvati et al., where transferrin-targeted silica nanoparticles also experienced a reduction in binding specificity after exposure to serum.[11]

Once we had confirmed that serum protein effectively reduced the targeting specificity of Herceptin-conjugated nanoparticles towards ErbB2+ cells, we determined whether

backfilling the nanoparticles with thiolated PEG rescues the targeting specificity of the nanoparticles after exposure to serum protein. PEG groups are known to reduce nonspecific protein adsorption.[5] We hypothesize that nucleation of the protein corona occurs when there are available sites on the nanoparticle for the binding of serum proteins, which can lead to the growth of the corona. To address this problem, we saturated the remaining surface of the nanoparticles with thiol-terminated PEG groups, followed by incubation with human serum. The thiolated PEG is datively bound to the nanoparticle surface because of the strong binding strength of sulfur to gold atoms. For nanoparticles that do not permit thiol–metal interactions, other linking chemistry such as click or carbodiimide chemistry may be used for backfilling the nanoparticle surface with PEG.[18] PEG backfilling effectively suppressed the adsorption of serum proteins to the surface of the nanoparticles (Figures S5B, S7).

We used a 2 kDa thiol-terminated mPEG for backfilling the Herceptin-conjugated gold nanoparticles. When introduced to cells, these backfilled nanoparticles were shown to be 2.5-fold more responsive to SKBR3 cells than MCF7 cells, with specificity indices of 3.07 and 1.24 (Figure 1 E), respectively. This result suggests that mPEG backfilling can partially rescue the specificity of the nanoparticles for ErbB2 receptors after exposure to serum.

Next, we wanted to evaluate whether the chain length, which is a function of the molecular weight, of the backfilled linear PEG molecule is important for determining the specificity of the Herceptin-conjugated nanoparticles for ErbB2+ cells. We produced particles backfilled with PEG of molecular weight 1, 5, and 10 kDa. We first assessed the binding specificity of these nanoparticles prior to serum exposure and found that nanoparticles backfilled with 1 kDa PEG displayed similarly high binding specificity for SKBR3 cells when compared to 2 kDa backfill, while larger PEG (5 and 10 kDa) produced a drastic loss in binding specificity (Figure 2 A, B). Upon exposing these nanoparticles to human serum, all of the PEG groups (1, 2, 5, and 10 kDa) used to backfill the nanoparticle surface showed a similar capacity to reduce serum-protein adsorption (Figure S5B). Qualitative (Figure S5C) and quantitative (Table S1) determination of ligand density on these nanoparticles showed similar amounts of conjugated Herceptin per particle, thus suggesting that the ligands are stable on the gold nanoparticle surface and that differences in the binding specificity are attributed to PEG length. Furthermore, nanoparticles backfilled with low molecular weight PEG were able to retain high specificity for ErbB2+ cells, while this specificity is lost when larger PEG groups are used. Since a 5 kDa linker was used for the conjugated Herceptin, anything equivalent or higher can potentially sterically hinder the Herceptin from binding to the target. This suggests that although the binding specificity can be improved through PEG backfilling, it is also important to choose the backfill length based on the ligand linker length.

While it is known that PEG is an effective antifouling polymer for nanoparticles, a better understanding of the PEGylation process is key to ensuring that the surface chemistry is appropriate for biomedical use. Although PEG can facilitate the reduction of nonspecific binding to cancer cells, this process is not as simple as putting PEG on the surface, since the chain length of the backfilled PEG has to be less than that of the ligand linker in order to not interfere with the binding of the biorecognition molecule to its target receptor (Figure 2 C). PEG backfilling reduces the number of serum proteins that bind to the nanoparticle surface and prevents these proteins from sterically blocking the binding of the biorecognition molecule to the receptor target. This strategy improves the specificity of the nanoparticle conjugates and allows them to be used for selectively recognizing diseased cells in a serum rich environment.

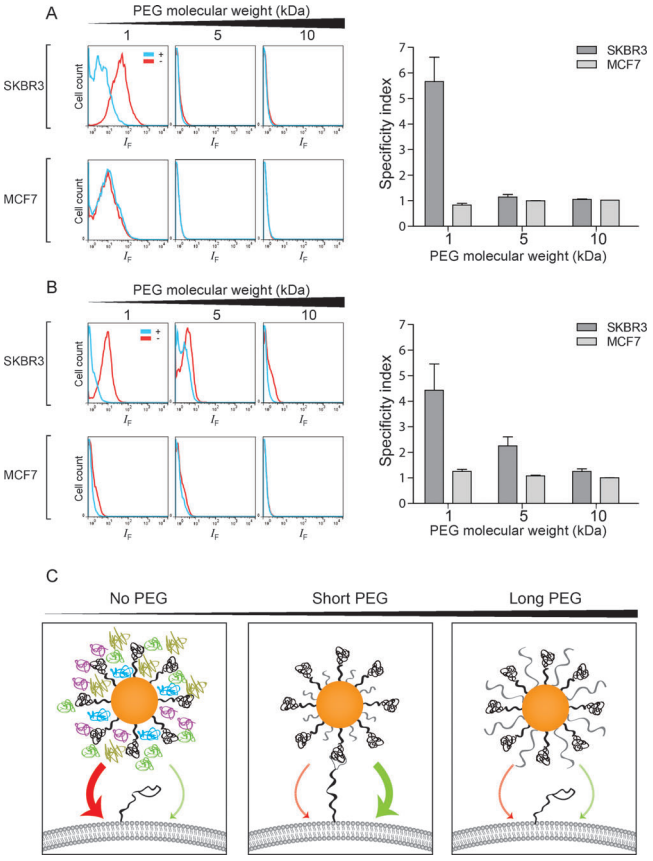

Figure 2. The molecular weight of the PEG used for backfilling affects the binding specificity. Herceptin-conjugated gold nanoparticles with 1, 5, and 10 kDa PEG backfill were incubated with SKBR3 and MCF7 cells prior to (A) and post (B) exposure to human serum. I_F = fluorescence intensity, cell count = normalized number (all events included) of cells counted. Red and blue lines represents cells incubated in the absence and presence of competitive Herceptin molecules, respectively. Error bars represent the standard error, where $n = 3$. C) The binding specificity is dependent on serum-protein adsorption and the molecular weight of the PEG used for backfilling. When the nanoparticles are exposed to a serum-rich environment, the surface coverage of the PEG backfilling and serum-protein adsorption determines the targeting specificity of the nanoparticle. The size of the arrows indicates the likelihood of interaction of the nanoparticles with the cells, with red indicating an alternative (non-ErbB2) pathway and green indicating the ErbB2-mediated pathway.

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[1] a) M. E. Davis, Z. G. Chen, D. M. Shin, *Nat. Rev. Drug Discovery* **2008**, *7*, 771 – 782; b) D. L. J. Thorek, A. K. Chen, J. Czupryna, A. Tsourkas, *Ann. Biomed. Eng.* **2006**, *34*, 23 – 38; c) D. G. Georganopoulou, L. Chang, J. Nam, C. S. Thaxton, E. J. Mufson, W. L. Klein, C. A. Mirkin, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 2273 – 2276; d) I. L. Medintz, H. T. Uyeda, E. R. Goldman, H. Mattoussi, *Nat. Mater.* **2005**, *4*, 435 – 446.

[2] a) D. B. Kirpotin, D. C. Drummond, Y. Shao, M. R. Shalaby, K. Hong, U. B. Nielsen, J. D. Marks, C. C. Benz, J. W. Park, *Cancer Res.* **2006**, *66*, 6732 – 6740; b) G. Wu, W. Yang, R. F. Barth, S. Kawabata, M. Swindall, A. K. Bandyopadhyaya, W. Tjarks, B. Khorsandi, T. E. Blue, A. K. Ferketich, et al., *Clin. Cancer Res.* **2007**, *13*, 1260 – 1268.

[3] a) J. D. Hood, M. Bednarski, R. Frausto, S. Guccione, R. A. Reisfeld, R. Xiang, D. A. Cheresh, *Science* **2002**, *296*, 2404 – 2407; b) D. Simberg, T. Duza, J. H. Park, M. Essler, J. Pilch, L. Zhang, A. M. Derfus, M. Yang, R. M. Hoffman, S. Bhatia, et al., *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 932 – 936.

[4] a) O. C. Farokhzad, S. Jon, A. Khademhosseini, T. N. T. Tran, D. A. Lavan, R. Langer, *Cancer Res.* **2004**, *64*, 7668 – 7672; b) V. Bagalkot, L. Zhang, E. Levy-Nissenbaum, S. Jon, P. W. Kantoff, R. Langer, O. C. Farokhzad, *Nano Lett.* **2007**, *7*, 3065 – 3070.

[5] a) A. S. Pitek, D. O'Connell, E. Mahon, M. P. Monopoli, F. Baldelli Bombelli, K. A. Dawson, *PloS one* **2012**, *7*, e40685; b) C. D. Walkey, J. B. Olsen, H. Guo, A. Emili, W. C. W. Chan, *J. Am. Chem. Soc.* **2012**, *134*, 2139 – 2147.

[6] a) C. H. J. Choi, C. A. Alabi, P. Webster, M. E. Davis, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1235 – 1240; b) R. K. Jain, T. Stylianopoulos, *Nat. Rev. Clin. Oncol.* **2010**, *2*, 653 – 664; c) A. Albanese, A. K. Lam, E. A. Sykes, J. V. Rocheleau, W. C. W. Chan, *Nat. Commun.* **2013**, *4*, 2718.

[7] a) W. Jiang, B. Y. S. Kim, J. T. Rutka, W. C. W. Chan, *Nat. Nanotechnol.* **2008**, *3*, 145 – 150; b) P. Decuzzi, M. Ferrari, *Biomaterials* **2007**, *28*, 2915 – 2922.

[8] a) I. Lynch, K. A. Dawson, *Nano today* **2008**, *3*, 40 – 47; b) M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, K. A. Dawson, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 14265 – 14270.

[9] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, et al., *Nat. Nanotechnol.* **2013**, *8*, 772 – 781.

[10] a) A. Salvati, A. S. Pitek, M. P. Monopoli, K. Prapainop, F. B. Bombelli, D. R. Hristov, P. M. Kelly, C. Åberg, E. Mahon, K. A. Dawson, *Nat. Nanotechnol.* **2013**, *8*, 137 – 143; b) V. Mirshafiee, M. Mahmoudi, K. Lou, J. Cheng, M. L. Kraft, *Chem. Commun.* **2013**, *49*, 2557 – 2559.

[11] a) R. Gref, M. Lück, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R. Müller, *Colloids Surf. B* **2000**, *18*, 301 – 313; b) K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, *Angew. Chem.* **2010**, *122*, 6430 – 6452; *Angew. Chem. Int. Ed.* **2010**, *49*, 6288 – 6308; c) A. Vonarbourg, C. Passirani, P. Saulnier, J.-P. Benoit, *Biomaterials* **2006**, *27*, 4356 – 4373.

[12] S. D. Perrault, W. C. W. Chan, *J. Am. Chem. Soc.* **2009**, *131*, 17042 – 17043.

[13] M. C. Daniel, D. Astruc, *Chem. Rev.* **2004**, *104*, 293 – 346.

[14] G. F. Paciotti, L. Myer, D. Weinreich, D. Goia, N. Pavel, R. E. McLaughlin, L. Tamarkin, *Drug Delivery* **2004**, *11*, 169 – 183.

[15] C. A. Hudis, *N. Engl. J. Med.* **2007**, *357*, 39 – 51.

[16] L. Y. T. Chou, W. C. W. Chan, *Adv. Healthcare Mater.* **2012**, *1*, 714 – 721.

[17] N. Chattopadhyay, Z. Cai, J. Pignol, B. Keller, E. Lechtman, R. Bendayan, R. M. Reilly, *Mol. Pharm.* **2010**, *7*, 2194 – 2206.

[18] R. A. Sperling, W. J. Parak, *Philos. Trans. R. Soc. London Ser. A* **2010**, *368*, 1333 – 1383.

