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Ligand Density and Nanoparticle Clustering Cooperate in the Multivalent Amplification of Epidermal Growth Factor Receptor Activation

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Abstract

Multivalent presentation of ligands on nanoparticles (NP) is considered a general strategy for enhancing receptor binding and activation through amplification of ligand-receptor interactions within the footprint of the NP. The spatial clustering of ligand-functionalized NP represents an additional, less well understood mechanism for increasing local ligand-receptor interactions, especially for receptors that form higher order assemblies, such as the epidermal growth factor (EGF) receptor (EGFR). To shed light on the interplay between ligand density (*i.e.* multivalency) and NP clustering in signal amplification, we apply EGF-functionalized 72 ± 1 nm gold nanoparticles (NP-EGF) with known ligand loading (10 – 200 EGF/NP) as quantifiable and experimentally tractable units of EGFR activation and characterize the NP-mediated amplification of EGFR phosphorylation as function of both EGF surface density and NP-EGF clustering for two cancer cell lines (HeLa and MDA-MB-468). The measurements confirm a strong multivalent amplification of EGFR phosphorylation through NP-EGF on the cellular level that results in EGF-loading-dependent maximum EGFR phosphorylation levels. A microscopic analysis of NP-EGF-induced EGFR phosphorylation reveals a heterogeneous spatial distribution of EGFR activation across the cell surface. Clustering of multivalent NP-EGF on sub-diffraction limit length scales is found to result in a local enhancement of EGFR phosphorylation in signaling “hot-spots” from where the signal can spread laterally in an EGF-independent fashion. Increasing EGF loadings of the NP enhance NP-EGF clustering and intensify EGFR phosphorylation. These observations suggest that NP-EGF-clustering and the associated local enhancement of ligand-receptor interactions are intrinsic components of the multivalent amplification of phosphorylation for the heterogeneously distributed EGFR through NP-EGF.

Keywords

epidermal growth factor receptor; gold nanoparticles; cell signaling; nanoparticle-cell interactions; multivalency

Supporting Information: Supporting Figures S1–S6 contain the gel electrophoretic characterization of NP, intracellular NP distribution determined by FIB-SEM, darkfield images of NP binding in the presence and absence of ligand, SEM images of NP labeled cells, number of EGF bound per NP, EGF ELISA calibration data. Table S1 summarizes scattering intensities for NP-EGF and Supporting Movies M1–M3 contain FIB-SEM data. The supporting information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

EGFR is a transmembrane receptor and a model receptor tyrosine kinase (RTK). EGF binding to the extracellular domain of its receptor induces a configurational change that allows receptor dimerization and subsequent cross-phosphorylation.^{1–4} Effector molecules recognize the phosphorylated receptor and initiate subsequent signaling events. In cellular systems the signaling outcome is, however, subject to additional regulation mechanisms. For instance, the local signal intensity has to overcome a noise threshold to result in a measurable cellular effect.^{5,6} In the case of EGFR, lateral interactions between activated and non-activated receptors facilitate a signal amplification through lateral propagation of EGFR activation.^{7–11} Of particular interest from the perspective of nanomedicine is that the multivalent presentation of EGF ligands on NP (NP-EGF) provides an alternative strategy to amplify signal activation and transduction.¹² Multivalency¹³ increases the binding affinity of NP and, thus, ligand receptor-binding,^{14–18} which can impact uptake rate¹⁹ and mechanism.²⁰ Importantly, multiple ligand-receptor contacts within the footprint of a particle can generate a strong local receptor activation.^{12,17,21,22} Previous studies applied EGF-functionalized, micron-sized beads^{9,10} or surface patterns^{23,24} to investigate the relationship between the “local” multivalent EGFR activation and the global EGFR phosphorylation. These studies demonstrated that an increased local EGF density can – under specific conditions – trigger a massive lateral phosphorylation propagation, but the applied probes lacked the resolution to elucidate how the spatial membrane receptor compartmentalization on sub-diffraction limit length scales affects the initial phosphorylation and its lateral spread, and were too big^{25,26} for potential applications in nanomedicine.

EGFR is known to be heterogeneously distributed in the plasma membrane^{27,28} and to be locally enriched in surface compartments with typical sizes on the order of 150 nm.²⁹ This heterogeneity in the surface distribution of EGFR is expected to aid EGFR oligomerization by creating membrane domains with high local receptor concentrations. It has been demonstrated that EGFR oligomers (assemblies larger than dimers) play an important role in enhancing the local receptor phosphorylation after initial stimulation.^{28,30–32} A local enrichment of EGFR in the membrane due to receptor oligomerization or spatial confinement in a structured membrane will necessarily influence the spatial distribution of EGFR targeting NP-EGF with potential implications for signaling initiation due to a locally increased number of ligand-receptor contacts. The relationship and potential synergism between NP multivalency, NP clustering and signal amplification remains, however, insufficiently understood. To shed some light on these relationships and to elucidate potential cross-dependencies, this study characterizes EGFR phosphorylation as measure for signal initiation as function of both ligand density and NP clustering. Specifically, we apply EGF-functionalized gold nanoparticles (NP-EGF) with an approximate diameter of 72 nm and controlled EGF ligand densities as experimentally tractable stimulus of receptor activation. The binding of EGF-functionalized gold NP can be precisely measured with inductively coupled plasma mass spectroscopy (ICP-MS),³³ facilitating a quantitative characterization of the relationship between receptor phosphorylation and amount of bound ligand. Plasmonic NP also provide large cross-sections for optical detection.^{34–36} Although multiple NP in a diffraction-limited spot cannot be directly resolved in conventional optical microscopy, a spatial accumulation of NP results in a measurable increase in scattering intensity (“brightness”).³⁷ Furthermore, distance-dependent electromagnetic interactions

between individual gold NP provide additional cues about their spatial organization and separation. If the separation of the NP is below approximately one NP diameter, the localized surface plasmon resonance (LSPR)^{38,39} of the gold NP red-shifts due to distance-dependent plasmon coupling.^{37,40–45} Scattering intensities and wavelengths are quantifiable in the far-field,^{46–52} facilitating a systematic investigation of the spatial correlation between NP-EGF clustering and EGFR phosphorylation through quantitative optical darkfield microscopy. Alternative detection schemes based on differential interference contrast (DIC) have been developed, as well.^{53,54} Due to advantageous physico-chemical properties and the ease of surface functionalization, gold NP-EGF are ideal probes for elucidating the relationships between ligand density, spatial NP clustering and NP-EGF enhanced EGFR phosphorylation. We emphasize that although this work focuses on EGFR as target, the findings have broader relevance as receptor oligomerization and clustering play a role in the function of other receptors than RTK.

RESULTS AND DISCUSSION

NP Design and Characterization.

All experiments were performed with approximately 72 ± 1 diameter gold NP wrapped in a surface-assembled membrane containing the zwitterionic lipid dipalmitoylphosphatidylcholine (DPPC) and cholesterol as main components (Figure 1A and B). The size of the NP was chosen to maximize the optical scattering signal but still allow binding of multiple NP to EGFR-enriched membrane domains. The zwitterionic membrane coat around the NP provides a thin but efficient passivation layer of the NP surface and stabilizes the NP under the chosen experimental conditions.^{56–60} The lipid layer was self-assembled around the gold NP in the presence of octadecanethiol, which binds covalently to the gold surface and generates a hydrophobic surface onto which lipids can assemble.^{61,62} To facilitate the covalent attachment of EGF to the NP, we also included azide-terminated 1,2-Diastearoyl-*sn*-glycero-3-phosphoethanolamine(DSPE)-polyethylene glycol(PEG-2000) into the lipid mix to allow the introduction of alkyne-functionalized EGF using the Cu^I-catalyzed dipolar cycloaddition.⁶³ The DSPE-PEG-2000-azide provides a PEG linker of 45 ethylene glycol units between the EGF and the NP surface with a contour length of approximately 19 nm and a persistence length of 0.37 nm.^{64,65} The end-to-end distance of the linker is predicted to be 3.7 nm based on the worm-like-chain estimate.⁶⁶ The conformational flexibility and contour length of the spacer linked to the compact lipid wrap provides the tethered EGF with some degree of conformational freedom.^{67–69} We generated NP-EGF with average EGF loadings in the range between 10 – 200 EGF/NP (Figure 1C), corresponding to average ligand densities of approximately 1 EGF / 2000 nm² to 1 EGF / 100 nm². Based on the simple geometric model outlined in Figure 1D we estimate that these densities translate to a maximum number of approximately 1 and 25 EGF ligands, respectively, in the contact area between NP-EGF and cell surface. Figure 1E and F summarizes the hydrodynamic diameters and zeta potentials of the NP before and after assembly of the membrane wrap as well as after EGF binding for different EGF surface densities. The successful membrane assembly is indicated by an increase in the hydrodynamic diameter of at least 3 nm and a less negative zeta-potential. The latter is due to the zwitterionic nature of the lipids that generate a lower surface charge than the initial

citrate ligands. Binding of EGF results only in a marginal increase in hydrodynamic diameter as the partial coverage of the surface with PEG-azide linkers bound to DSPE lipids has already expanded the hydrodynamic diameter; the small (~2.5 nm) EGF is accommodated within this PEG layer. Binding of EGF to the NP is confirmed by a decrease in zeta potential relative to the membrane-wrapped NP and by changes in gel electrophoretic mobility of the NP (Figure S1). The UV-Vis spectra in Figure 1G show a spectral red-shift of LSPR after the membrane assembly step, which is consistent with an increase in refractive index in the immediate vicinity of the NP due to successful membrane wrapping. Loading of EGF onto the PEGs has a much weaker effect and only leads to an additional discernable spectral shift for the high EGF density case with 200 EGF/NP.

Cellular NP-EGF Distribution and Multivalent Enhancement of NP Binding.

We chose HeLa and MDA-MB-468 cancer cells to study NP-EGF-induced binding in this work as these cells represent well-characterized models for cells with physiological (HeLa) or highly increased (MDA-MB-468) EGFR expression levels.⁷⁰ As EGFR signaling is spatially regulated, EGFR phosphorylation at the plasma membrane can lead to different outcomes than cytoplasmic signaling associated with endosome containing phosphorylated EGFR (pEGFR).^{71,72} Knowledge of the cellular distribution of NP-EGF activated EGFR is, thus, important to understand the underlying signaling processes. We imaged the cellular distribution of NP-EGF in MDA-MB-468 cells after incubating with a 30 pM solution of NP-EGF (100 EGF/NP) for $t_{\text{inc}} = 15, 30, 60$ min through combined focused ion beam milling and scanning electron microscopy (FIB-SEM, Figure 2A and Supporting Movies M1–M3). Figure 2B shows an individual cell before milling and Figure 2C summarizes the resulting depth distribution profiles for the three investigated incubation times (t_{inc}). Representative SEM images after 5 s and 45 s of ion milling for cells incubated with NP-EGF for $t_{\text{inc}} = 15, 30, 60$ min (top, middle, bottom) are summarized in Figure 2D. The depths profiles reveal that for $t_{\text{inc}} = 15$ min NP-EGF are primarily associated with the cell surface (68% of the NP are within one particle diameter of the cell surface), but that with increasing time, the NP are uptaken and distributed throughout the cytoplasm. Since we focus in this work on plasma-membrane localized EGFR activation in living cells, we limited the incubation time with NP-EGF to 10 min and subsequently immediately fixed the cells to ensure that the overwhelming majority of the NP-EGF are bound but not yet internalized and that the measured phosphorylation is primarily associated with the plasma membrane. Based on the progression of the uptake established in Figure 2C, we project that at least 75% of NP-EGF are contained within 80 nm (~1 NP diameter) of the cell surface at $t_{\text{inc}} = 10$ min (Figure S2). Compared with alternative pharmaceutical approaches that block receptor internalization at physiological temperatures, the chosen approach of limiting the incubation time minimizes cellular stress and avoids undesirable side effects.

Figure 3A&B plots the number of NP-EGF per cell as function of NP input concentration [NP-EGF] for nanoconjugates as determined by inductively coupled mass spectroscopy (ICP-MS) with 10, 50, 100, or 200 EGF/NP for both HeLa and MDA-MB-468 cells after 10 min of incubation. For both MDA-MB-468 and HeLa the gold NP signal increases overall with input concentration and EGF loading, confirming a multivalent enhancement of NP-

EGF binding. Control experiments with membrane wrapped NP without EGF confirmed that the NP binding was EGF-dependent (Figure S3).

Amplification of EGFR Phosphorylation through Multivalent EGF Presentation.

NP-EGF binding induces EGFR phosphorylation, which can further spread through EGFR-EGFR-mediated transactivation. We quantified EGFR phosphorylation at Tyr1086 as function of EGF surface loading by immunolabeling and subsequent flow cytometry analysis for HeLa and MDA-MBA-468. The measured EGFR phosphorylation *versus* NP-EGF concentration plots show a logarithmic growth behavior for all EGF loadings (Figure 3C and D). Importantly, for both cell lines the efficacy (maximum phosphorylation) and potency (concentration corresponding to $\frac{1}{2}$ of the maximum phosphorylation) of NP-EGF increases with increasing EGF loading. Consistent with the differences in receptor expression levels, the phosphorylation measured for EGFR-overexpressing MDA-MB-468 exceeds that of HeLa. To compare the phosphorylation obtained for NP-EGF with that of free EGF we calculated the effective EGF concentrations for the nanoconjugates by multiplying the EGF surface loading with the NP concentrations. In Figure 3E and F we compare the measured phosphorylation levels for NP-EGF with EGF loadings of 50 EGF/NP, 100 EGF/NP, or 200 EGF/NP at effective EGF concentrations of 1.5, 3.0, or 6.0 nM with each other as well as with free EGF of the same concentration. EGF loadings > 50 EGF/NP systematically yield a stronger phosphorylation levels than free EGF. The important role of the EGF density in determining cellular phosphorylation levels is evidenced by the observation that the differences in phosphorylation for NP-EGF with different EGF loading (differences within one color) are larger than those for NP-EGF with identical EGF loading but different effective EGF concentrations (differences between colors). It is interesting that NP-EGF with 50 EGF/NP show less phosphorylation than free EGF at the same effective EGF concentration despite the fact that even the initial NP-cell contact area (Figure 1D) contains more than one (~ 6) EGF to bind to EGFR on the cell side. The lower phosphorylation when compared with free ligand could result from a decrease in the binding affinity of the individual EGF after tethering to the PEG spacer⁷³ or from other perturbations that are associated with conjugation to NP (slower diffusion, steric hindrance, *etc.*) and that need to be compensated by a higher degree of multivalency.

Although the data in Figure 3E and F generally confirm a positive correlation between phosphorylation and EGF loading, one caveat in comparing phosphorylation levels of NP-EGF with different EGF loadings as function of NP-EGF input is that it does not provide sufficient information to differentiate between effects associated with an intrinsic gain in EGFR phosphorylation efficacy and binding affinity of the NP. Different EGF surface densities influence the binding (see Figure 3A and B) and, thus, the total number of delivered NP-EGF.^{74–77} To eliminate trivial effects in phosphorylation that arise from differences in NP binding, we compared the phosphorylation obtained for NP-EGF with different EGF loadings at identical concentrations of delivered EGF. Figure 3G and H plots the phosphorylation as function of the number of delivered EGF for MDA-MB-468 and HeLa. This number was obtained by multiplying the number of delivered NP per cell determined by ICP-MS with the EGF surface loading determined by ELISA. The graph shows that NP-EGF with higher EGF loading deliver more EGF but that in the concentration

ranges where NP-EGF with different EGF densities overlap, the nanoconjugates with higher EGF loading still achieve higher EGFR phosphorylation even though the number of delivered EGF is identical.

Intriguingly, NP-EGF with different EGF loadings do not converge against a common maximum phosphorylation level in the asymptotic limit (Figure 3G and H). Instead, the maximum phosphorylation increases with growing EGF loading in the sequence $10 \text{ EGF/NP} < 50 \text{ EGF/NP} < 100 \text{ EGF/NP} < 200 \text{ EGF/NP}$. Different efficacies imply a non-linear amplification of EGFR phosphorylation with increasing EGF density and reveal that the effect of a lower EGF surface density on the maximum phosphorylation cannot be off-set by an increase in NP-EGF input. Multivalency is expected to increase the binding of EGF-functionalized NP, but a higher binding affinity alone cannot account for an increase of the *maximum* cellular phosphorylation. Instead, the increase of the asymptotic phosphorylation with growing ligand indicates a qualitatively different receptor activation mechanism in response to increasing EGF densities.

We validated the phosphorylation trends by flow cytometry through independent quantitative Western Blots. Figure 4A contains immunoblot images of pEGFR and EGFR for MDA-MB-468 and HeLa, respectively, and Figure 4B and C summarizes the relative EGFR phosphorylation intensities normalized by the total EGFR protein concentration for the investigated NP-EGF conditions. The Western Blots show again an increase in EGFR phosphorylation as function of both EGF loading and NP input for HeLa and MDA-MB-468. The phosphorylation obtained for the supernatant of 60 pM NP-EGF with 200 EGF/NP (included as control in Figure 4A) was low, confirming that the phosphorylation gains in Figure 4B and C were caused by NP-EGF, not residual free EGF.

Spatial Mapping of NP-EGF-Induced EGFR Phosphorylation.

The data in Figures 3 and 4 confirm a multivalent amplification of phosphorylation through NP-EGF with EGF loadings $> 50 \text{ EGF/NP}$. To elucidate the spatial relationship between NP-EGF binding and EGFR phosphorylation and to account for differences in the asymptotic phosphorylation levels, we combined conventional confocal fluorescence microscopy of immunolabeled pEGFR and confocal backscattering microscopy of cell-bound NP. As benchmark we first imaged the spatial distribution of pEGFR in the dorsal plasma membrane after incubating cells with 3 nM free EGF for 10 min. The fluorescence induced by EGF is not homogeneously distributed, instead the confocal scans show discrete “spots” of increased fluorescence intensity as illustrated in Figure 5A for MDA-MB-468. This pEGFR distribution is consistent with the activation of a heterogeneously distributed EGFR receptor that results in a spatial enrichment of EGFR in membrane domains.^{6,11,78,79} An additional important baseline for evaluating the effect of EGF nanoconjugation and pEGFR distribution is the effect of residual free ligand in the washed NP-EGF preparations. The supernatant of a 30 pM NP-EGF preparation with 200 EGF/NP shows only a weak activation limited to specific spots (Figure 5B).

In the next step, we mapped the spatial distribution of pEGFR induced by NP-EGF with high (200 EGF/NP) and low (50 EGF/NP) ligand density. We incubated the cells again with NP-EGF at concentrations of 5, 15, 30 pM for 10 min. Figure 5C and D shows confocal

scans obtained with MDA-MB-468 cells after treatment with a 15 pM solution of NP-EGF and surface loadings of 50 and 200 EGF/NP, respectively. The individual panels contain the backscattering scan that maps the NP-EGF distribution, the fluorescence scan that maps the pEGFR distribution, a merger of the latter two, and a computed map highlighting pixels that contain intensities on both channels. To quantitatively characterize the spatial relationship between NP-EGF binding and EGFR phosphorylation, we calculated Manders Overlap Coefficients (MOC).⁸⁰ The latter measures the fraction of pixels that have a detectable signal intensity in both monitored signal channels, independent of the magnitude of the signal.⁸¹ We chose the MOC approach to evaluate the spatial overlap between NP and pEGFR signal as the intensities of these two species can vary significantly considering that different signals are detected (resonant backscattering *versus* fluorescence). A MOC value of 1 corresponds to perfect colocalization, whereas a value of 0 indicates absence of colocalization. M1 quantifies the overlap of backscattering signal with fluorescence and M2 the overlap between fluorescence and backscattering. The calculated M1, M2 values for all experimental conditions in MDA-MB-468 are summarized in Figure 5E. For 200 EGF/NP, M1 is systematically higher than M2 for all NP-EGF input concentrations. The M1 values increase with NP-EGF input and lie in the range 0.32 ± 0.04 to 0.50 ± 0.04 . The maximum M1 colocalization value is consistent with a NP-EGF-induced phosphorylation but also shows that not all NP-EGF binding events are successful in inducing a measurable phosphorylation signal, presumably due to existing cellular inactivation mechanisms.^{82, 83} Furthermore, not all backscattering signals are caused by NP-EGF as larger cellular structures can also contribute to the detected scattering signal so that a perfect colocalization is not expected. Importantly, the M2 values remain below 0.26 ± 0.03 for all NP-EGF concentrations. In the case of 50 EGF/NP, both M1 and M2 remain < 0.20 due to an overall weak NP-induced EGFR phosphorylation. We performed an identical set of imaging experiments with HeLa cells and the resulting M1 and M2 values are summarized in Figure 5F. The MOC values show the same trends as observed for MDA-MB-468, but the M1 values for 200 EGF/NP lie lower (between 0.22 ± 0.03 and 0.44 ± 0.07) than for the EGFR overexpressing MDA-MB-468.

The systematic difference between M1 and M2 for NP-EGF with high ligand loading in Figure 5E and F is intriguing. Increased M1 values paired with low M2 values indicate that NP-EGF containing regions show significant pEGFR phosphorylation, but that the pEGFR signal also exists in areas void of NP-EGF. Part of this non-colocalizing fluorescence intensity can be attributed to background phosphorylation as observed in Figure 5B. However, it is noteworthy that NP-EGF binding often induces EGFR phosphorylation in direct vicinity of the NP, resulting in NP-EGF being embedded in larger pEGFR-positive regions, as shown in the magnified cell sections in Figure 5C. This behavior is characteristic of a lateral propagation of activation around initial NP-EGF binding sites. Different from previous studies that reported a rapid and extensive propagation of receptor phosphorylation over entire cell after local activation with EGF-functionalized multivalent beads or microfluidic flow,^{8,9} the spread induced by the smaller NP-EGF observed in this work is not global but more limited to the region around the NP. Intriguingly, this spread occurs in cells with high (MDA-MB-468) or physiological (HeLa) receptor expression levels, provided the EGF loading of the NP are sufficiently high. We attribute this behavior to the heterogeneous

spatial distribution of EGFR, which generates sufficiently high regional EGFR densities in the plasma membrane to facilitate a spatially limited lateral phosphorylation transfer in both HeLa and MDA-MB-468 cells.

NP-EGF Clustering and EGFR Phosphorylation: Indications of Signaling “Hot Spots:

Scanning electron microscopy images of NP-EGF-treated cells show a significant clustering of the NP on the cell surface (Figure S4). To quantify the relationship between NP-EGF concentration and EGFR phosphorylation at the location of NP-EGF binding, we integrated the pEGFR fluorescence signal over the area defined by surface-bound NP-EGF and quantified the resulting fluorescence as function of NP intensity. We normalized the backscattering signal of the NP by dividing through the average intensity of an individual NP. Although electromagnetic coupling between NP (*vide infra*) affects signal intensities,³⁷ the normalized confocal backscattering intensities give an acceptable first estimate for the number of NP per investigated spot. Figure 6A–D summarizes the measured pEGFR fluorescence as function of normalized NP intensity for different input concentrations of NP-EGF with ligand densities of 50 EGF/NP or 200 EGF/NP for HeLa (A, B) and MDA-MB-468 (C, D). The associated Pearson correlation coefficients (PCC) for the relationship between fluorescence and backscattering intensities and their statistical p-values (two-sided Student t-test) are included in Figure 6E. The local NP scattering signal, or NP clustering, in Figure 6A–D increases with NP-EGF input and EGF loading and is in general higher for MDA-MB-468 than for HeLa. The average number of NP-EGF contained per diffraction-limited spot for the measured distributions are summarized in Table S1. At conditions that favor NP-EGF clustering on the cell surface, *i.e.* high NP-EGF input concentrations (30 pM) and high ligand densities (200 EGF/NP), a strong positive correlation between pEGFR signal, measured as fluorescence intensity, and NP-EGF signal is observed. The peak NP and fluorescence intensities as well as the correlation are higher for the EGFR over-expressing MDA-MB-468 (PCC = 0.82) than for HeLa (PCC = 0.67). For the low EGF density case, NP-EGF clustering at high NP-EGF input concentration still achieves a moderate positive correlation between local fluorescence intensity and NP-EGF signal with PCC = 0.62, 058 (MDA-MB-468, HeLa).

The findings that NP-EGF clustering increases with input concentration and EGF loading and that this gain in clustering is associated with a gain in EGFR phosphorylation has important implications for the interpretation of the global increase in EGFR phosphorylation as function of EGF loading and concentration in Figures 3 and 4. The data in Figure 6 reveal that the increase in cellular phosphorylation is not simply a result of more randomly distributed NP-EGF binding with identical phosphorylation response. Instead, the total signal is enhanced by formation of NP-EGF clusters that provide stronger EGFR phosphorylation.

The confocal analysis is limited in resolution by the wave nature of light. To identify the length scale of NP-EGF clustering that is relevant for the phosphorylation enhancement, we next characterized the fluorescence signal as function of the plasmon resonance wavelength of the NP, which encodes information about NP separations on the nanometer to tens of nanometer length scale. To that end, we collected confocal fluorescence scans of

fluorescently labeled pEGFR and darkfield scattering spectra maps of NP-EGF (200 EGF/NP) of the same field of view collected in a wide-field microscope. Figure 7A contains an example with the fluorescence scan on the top and a map of the fitted peak wavelength on the bottom. We point out that the differences in the ranges of the x,y-axes derive from the different pixelations of the used detectors. It is clear from the detected peak wavelengths that the plasmon resonances of NP-EGF bound to the cell surface are strongly red-shifted when compared with the same particles deposited on glass (Figure 7B). As glass has a similar refractive index ($n_r = 1.52$) as the cell surface, we attribute these large spectral shifts to a strong electromagnetic coupling between closely spaced NP. This finding is entirely consistent with findings from Abulrob *et al.* and others who have shown that EGFR in the plasma membrane of HeLa cells is locally enriched in surface compartments with typical dimensions on the order of 150 nm.²⁹ Binding of multiple NP to domains of this size would result in a crowding of NP and facilitate plasmon hybridization.⁸⁴ The magnitude of the spectral shift of these clusters increases with growing number of coupling NP and decreasing interparticle separation.³⁷

The positive correlation between LSPR wavelength and co-localized pEGFR signal intensity in Figure 7C confirms that NP-EGF association on deeply subdiffraction limit length scales is a key driver for NP-EGF-clustering-amplified EGFR phosphorylation. Importantly, the increase in phosphorylation as function of resonance wavelength is non-linear but becomes stronger with longer wavelength. Large NP clusters with short interparticle separations that achieve a strong shift of the plasmon resonance are most efficient in boosting EGFR phosphorylation.

Overall our analysis of Figures 6 and 7 confirms increasing levels of local phosphorylation due to spatial NP-EGF clustering. The strong increase in local phosphorylation associated with clustering accounts naturally for the differences in maximum phosphorylation observed for different EGF loading ratios in Figure 3G and H. Our data reveal that NP-EGF clustering induces the formation of sub-diffraction limit phosphorylation “hot-spots” which can enhance the phosphorylation response on a cellular level. As NP-EGF clustering increases with ligand loading (Figure 6, Table S1),^{17,21} one must conclude the local signal enhancement through NP-EGF clustering is an intrinsic component of signal amplification through multivalent NP for a heterogeneously distributed receptor.

CONCLUSION

We have investigated the global cellular EGFR phosphorylation intensity and its spatial distribution in HeLa and MDA-MB-468 cells as function of both NP-EGF input concentration and EGF loading in the range of 10 – 200 EGF/NP. On the cellular level the EGFR phosphorylation generally increases with NP-EGF input. Intriguingly, the maximum phosphorylation depends on the multivalency of the NP and increases with EGF loading. This behavior is insufficiently explained by a multivalent amplification of binding affinity alone and, instead, indicates a qualitative change in the receptor activation associated with increasing ligand loading of the NP. A quantitative analysis of the spatial correlation between local NP-EGF binding and EGFR phosphorylation reveals that NP-EGF-induced EGFR phosphorylation depends on the spatial distribution of the nanoconjugated EGF and

that NP-EGF clustering is an important parameter that influences maximum cellular phosphorylation levels. NP-EGF clusters induce locations of increased EGFR phosphorylation, and the intensity of these signaling “hot spots” increases with local NP-EGF concentration and EGF loading. The formation of clusters of nearly touching NP is particularly efficient for EGFR activation. EGFR phosphorylation is, however, not restricted to locations containing bound NP-EGF. We reconcile the increase in NP-EGF dependent phosphorylation detected by flow cytometry and Western Blotting with M2 values that are consistently lower than M1 through an NP-EGF-independent spread of EGFR phosphorylation, similar to what was observed before for EGF-loaded beads.^{9,10} For NP-EGF this spread is not global but spatially confined to the vicinity of the NP, and both the EGFR overexpressor MDA-MB-468 as well as HeLa with physiological EGFR expression level show this regional spread of phosphorylation. We conclude that – despite a large difference in formal expression level – in both cell lines membrane domains exist in which the heterogeneously distributed receptor is sufficiently high concentrated to facilitate a lateral trans-phosphorylation. As the initial pEGFR signal at the site of the NP contact is positively correlated with NP-EGF clustering and the latter increases with ligand loading of the NP, the findings of this study demonstrate that the accumulation of EGF-EGFR contacts through spatial NP-EGF clusters is an intrinsic component of the amplification of EGFR activation through multivalent NP-EGF. The local density of formed EGF-EGFR contacts depends both on the clustering of NP-EGF in the membrane as well as the EGF loading of the individual NP. Our combined biochemical and imaging studies show that an increase in EGF-EGFR density due to clustering of multivalent NP-EGF is associated with an EGF-loading-dependent increase in the maximum EGFR phosphorylation level. This functional dependence can be attributed to the facts that a local enrichment of receptors favors interactions between activated and non-activated receptors, shifts equilibria towards oligomerization, and facilitates ligand-independent lateral spread of receptor activation. Receptor clustering also alleviates ligand rebinding^{85,86} and is crucial for some second messenger driven phosphorylation^{87,88} after an initial activation. All of these effects can in principle account for the increase of the EGF-loading-dependent asymptotic EGFR phosphorylation level, but the relative contributions from the individual effects remain to be quantified. Naturally, the degree of clustering of a multivalent NP on the cell surface depends not only on the NP concentration and its ligand density but also on the spatial distribution of the receptors in the membrane. Since the latter may vary between individual cells and cell lines, the spatial distribution of the receptor could result in different signaling in response to NP-EGF and, thus, provide opportunities for controlling cellular behavior with NP. In closing, we would like to note that our finding that a multivalent presentation of EGF on NP does not only enhance the binding affinity of the NP but also strengthens signaling-relevant proximity effects in EGFR clusters has mechanistic implications also for other RTK and potentially a broader range of transmembrane receptors. Our study underlines the importance of the spatial organization of receptors and ligands for key steps in signaling initiation. Conceptualized in a broader context our findings suggest that the nanoscale spatial distribution of ligands in the extracellular matrix or during cell-cell contacts can influence signaling outcomes and could, thus, be an important factor for regulating cell development and differentiation.

METHODS

Colloidal Au NP Synthesis.

Colloidal gold seeds were prepared by heating 20 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (0.26 mM, Sigma-Aldrich, wt 393.83 g), and adding 800 μL of sodium citrate dihydrate (34.00 mM, Sigma-Aldrich, wt 294.10 g) immediately after boiling. The gold seeds were then grown to an average size of 80 nm by adding dropwise 80 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution (1.00 mM), and 80 mL of a mixture of sodium citrate dihydrate (0.85 mM) and L-Ascorbic acid (2.84 mM, Sigma-Aldrich, wt 176.12 g). The gold solution was then boiled for 30 min and neutralized to pH 7 with a 10 mM aqueous solution of NaOH (Sigma-Aldrich, wt 40.00 g). Before use, the colloidal gold solution was washed by centrifugation (720g, 10 min) and resuspension in Milli-Q water.

Membrane wrapping of Au NP.

A total amount of 2 μmole of lipid mixture (Avanti Polar Lipids) was prepared by mixing DPPC (10 mM), cholesterol (10 mM), DSPE-PEG (2000) azide (3 mM), and DOPS (5 mM) in a clean 25 mL round-bottom flask. The solvent chloroform in the lipid mixture was removed by rotary evaporator. A uniformly thin lipid layer formed on the bottom of the flask, which was then further dried under vacuum overnight. HEPES buffer (20 mM, Sigma-Aldrich, wt 238.30 g) was added to the dried lipid layer (1 mL 10 mM HEPES buffer per 1 μmole lipid), and the mixture was sonicated for 5 min to form a clear liposome solution. For each 500 μL of liposome solution, 50 μL of Au NP pellet (2×10^{11} particles/mL) was added. Subsequently, 40 μL of 1-Octadecanethiol (8.72 mM, Sigma-Aldrich, wt 286.56 g) dissolved in ethanol was added. The final volume was brought to 1 mL with HEPES buffer. The mixed solution was then shaken for 5 h, 18 h or 24 h, respectively. The NP were subsequently washed three times through centrifugation (720g, 10 min) and re-suspended in HEPES buffer.

NP-EGF Preparation.

EGF Recombinant Human Protein (ThermoFisher, 1mg/mL, 1X PBS) was functionalized by adding 2% v/v of Propargyl-N-hydroxysuccinimidyl ester (Sigma-Aldrich, 0.44 M in DMSO, wt 225.20 g) and incubated on ice for 6 h. The functionalized EGF was then dialyzed in 1x PBS *via* D-Tube Dialyzers (Millipore) overnight to remove excessive NHS ester. The conjugation was completed by mixing 5 μL of functionalized EGF with membrane-wrapped NP (NP: EGF, 1:50000). The catalyst was prepared by mixing a 0.10 M solution of L-Ascorbic acid with a 5.10 mM solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1:1 v/v). And 3 μL of catalyst was added to the mixture of alkyne-functionalized EGF and azide-presenting NP. The final volume of the mixture was brought to 100 μL with 0.1x PBS. To generate NP-EGF with different EGF loading, membrane-wrapped NP with different wrapping times were used (Figure S5). The crosslinking reaction was maintained at room temperature overnight. Subsequently, excessive EGF and catalyst were removed by dialysis with Nuclepore Track-Etched Membranes (Whatman, Pore Size 0.03 μm , Diameter 25 mm) in 0.1x PBS overnight.

Nanoparticle Characterization.

The hydrodynamic radius and zeta potential of nanoparticles were measured by Zetasizer Nano ZS90 (Malvern, Worcestershire, UK). The sample for size measurement contained 1×10^{11} particles/mL in Milli-Q water. The sample concentration for zeta potential measurement was 1×10^7 particles/mL in 10mM NaCl (pH 7.0). The absorption spectra of particle solutions (1×10^{11} particles/mL) were acquired on an Agilent Cary 5000 UV/vis spectrometer. Milli-Q water was used for baseline correction. All spectra were normalized by dividing through the peak intensities. The number of EGF conjugated to NP was quantified with the EGF Human ELISA Kit (ThermoFisher) (Figure S6).

Cell Culture.

The MDA-MB-468 cell lines was purchased from ATCC® (HTB-132™). The HeLa cell line was a gift from Sam Thiagalingam at Boston University. The cells were cultured in advanced Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, and grown in an incubator at 37 °C, 5% CO₂ and 95% relative humidity. The cell seeding density was 1×10^5 cells/mL, and experiments were conducted when the cells reached 80% confluency. HeLa cells were starved in serum-free DMEM/F-12 medium for 24 h before use. EGFR-overexpressing MDA-MB-468 cells were starved in serum-free DMEM/F-12 medium containing 20 mM of the anti-oxidant N-acetylcysteine (NAC) for 24h before use.

Flow Cytometry Quantification of EGFR Phosphorylation.

Cells were plated in 12-well dishes as described above. The cells were stimulated with a fresh medium containing NP-EGF or EGF at different concentrations for 10 min. Then, the medium was removed, and cells were washed with HBSS buffer. The cells were detached with StemPro™ Accutase™ Cell Dissociation Reagent (ThermoFisher) for 10 min at 37 °C, and collected by centrifugation (300 g, 5 min). The cells were fixed with 4% (w/v) Pierce™ formaldehyde (ThermoFisher) for 15 min, and permeabilized with 0.1% (w/v) Triton™ X-100 for 10 min. The cells were then incubated with Phospho-EGFR (Tyr1086) antibody (0.5 µg/mL, ThermoFisher) for overnight at 4 °C. Subsequently, the cells were stained with an Alexa Fluor 647 conjugated secondary antibody (4 µg/mL, ThermoFisher) for 1 h at room temperature. The cells were washed three times with DPBS after each step of the fixation and labeling procedure to remove excess reagents. The fluorescence intensity distribution for each sample was measured with a BD LSRII flow cytometer and analyzed through Flowing Software 2. All fluorescence intensities are background corrected (intensity of cell control without treatment are subtracted) and the calculated difference is reported as FACS intensity.

ICP-MS Quantification of NP-EGF Binding.

NP-EGF were prepared and bound to cells as described above. The number of cells in a sample were determined by a cell counter. A solution (30 µL) of cells was dissolved with 800 µL of aqua regia. The mixture was then dried overnight at 55 °C and re-dissolved in 1 mL of HCl solution (2% v/v). Subsequently, the samples were diluted 10x with HCl

solution, and the gold concentration in the samples were quantified with a Thermo (VG) PlasmaQuad (PQ) ExCell inductively coupled plasma mass spectrometer.

Confocal Imaging.

Cells were plated in 50 mm glass bottom dishes with a gridded glass inset (MatTek, gridded 1.5 coverslips). The gridded coverslips have an alphanumeric pattern, and offer imaging of cells and the grid in the same focal plane. The cells were stimulated with a fresh medium containing NP-EGF or free EGF at different concentrations for 10 min. Then, the medium was removed, and cells were washed with HBSS buffer. The cells were fixed with 4% (w/v) Pierce™ Formaldehyde (ThermoFisher) for 15 min. Then, cells were permeabilized with 0.1% (w/v) Triton™ X-100 for 10 min. Subsequently, the cells were incubated with Phospho-EGFR (Tyr1086) antibody (0.25 µg/mL, ThermoFisher) for overnight at 4 °C. Subsequently, the cells were stained with an Alexa Fluor 647 functionalized conjugated antibody (2 µg/mL, ThermoFisher) for 1h at room temperature. The cells were washed three times with DPBS after each step of the fixation and labeling procedure to remove excess reagents. Samples were imaged with scanning confocal microscope (Olympus FV 1000) at 543 and 633 nm. The backscattering signals were obtained using the 543 nm excitation line.

Dark-Field Microscopy.

The cells were imaged in an inverted microscope (Olympus IX71) under dark-field illumination through a high numerical aperture (NA) oil condenser (NA= 1.2–1.4). The scattering light was collected through a 60x oil objective (NA = 0.65) and captured on an electron multiplying CCD (EMCCD, Andor Ixon⁺). The exposure time for each monochromatic image was 0.05 s, and the cycle time for a full set of ten images was 0.5 s. Digital color images were recorded under white-light illumination using an Olympus SP310 digital camera attached to the microscope through an eyepiece adapter.

Image Processing and Analysis.

Confocal backscattering and fluorescence images were analyzed by ImageJ.⁸⁹ The Costes⁹⁰ auto threshold algorithm was applied to background corrected images before MOC⁸⁰ were calculated using algorithms implemented in the software. To correlate fluorescence and backscattering images, the particle finder routine was applied to localize discrete regions of interest. For these regions the intensities on the fluorescence and backscattering intensities were obtained by multiplying the average intensity with the size of the regions.

Focused Ion Beam (FIB) Sample Preparation.

Cells were plated in a 1 cm × 1 cm silicon chip, and treated with 50 pM of NP-EGF in fresh DMEM/F-12. Then, the medium was removed, and cells were washed with HBSS buffer. The cells were fixed with 0.4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 h at room temperature. After washing three times in 0.1M sodium cacodylate buffer for 10 min each time, the cells were post-fixed with 2% osmium tetroxide in distilled water for 30 min at room temperature. And cells were washed three times in Milli-Q water for 10 min each. The cells were then dehydrated by incubation in a series of solutions with ascending ethanol concentration (50%, 70%, 95%, 100%) for 15 min

each and followed by three washing steps in 100% ethanol for 15 min each. Specimens were then washed three times in 100% acetone for 15 min each. Subsequently, samples were dried with Hexamethyldisilazane (HMDS) and acetone (1:1, v/v) for 10 min at room temperature, followed by overnight incubation in 100% HMDS. Samples were imaged using a Zeiss Supra40VP SEM at 10.0 kV and a working distance of 10 mm. The samples were subsequently milled and imaged with an FEI Quanta 3D FIB instrument. The milling was done with 30 kV, 0.5 nA Gallium ion beam with 10-s intervals.

Western Blot.

Cells were plated in 12-well dishes as described above. The cells were stimulated with a fresh medium containing NP-EGF or supernatant at different concentrations for 10 min. Then, the medium was removed, and cells were washed with HBSS buffer. Cells were harvested in lysis buffer (Cell Extraction Buffer, 1mM PMSF, 10% (v/v) protease inhibitor cocktail; ThermoFisher) on ice for 30 min. Unlysed cell fragments were removed by centrifugation at 13000 rpm for 5 min at 4 °C. Protein samples were separated by SDS-PAGE using NuPAGE 3–8% Tris-Acetate Protein Gels (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After transfer, the membrane was blocked with 5% milk in TBST for 30 min at 25 °C. The membrane was washed with TBST and immunoblotting was performed the following with the primary and secondary antibodies at the indicated dilutions in TBST: phosphor-EGFR (Tyr1068) antibody (Cell Singling Technology, #2234; 1:1000) at 4 °C overnight; EGF Receptor Antibody (Cell Singling Technology, #2232; 1:1000) at 4 °C overnight, and goat anti-rabbit IgG-HRP (Jackson ImmunoResearch, 111-035-144; 1:5000) for 1 hr at 25 °C. Blot imaging was developed with the ChemiDoc XRS+ system (Bio-Rad). The intensity of chemiluminescent image was quantified by ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

A. Schematic drawing of membrane-wrapped gold nanoparticle functionalized with EGF (NP-EGF) used in this work (left) and molecular structures and mol% of lipid components (right). The crystal structure of EGF is Protein Data Bank (pdb) structure 1JL9.⁵⁵ **B.** TEM image of NP-EGF. **C.** Number of EGF molecules bound per NP as determined by ELISA for the particles used in this study. **D.** Geometrical model to estimate NP surface area O that is sufficiently close to the cell surface to facilitate EGF binding to EGFR. The length of the EGFR - EGF-PEG contact is approximated as 10 nm. **E.** Hydrodynamic diameter and **F.** zeta potential for citrate stabilized gold NP (Au NP), membrane wrapped NP (MWP), and membrane wrapped NP after EGF functionalization (NP-EGF). Data are provided for NP-EGF with 50, 100, 200 EGF / NP. **G.** UV-Vis spectra for NP-EGF with 50, 100, 200 EGF/NP (top to bottom). The data presented in C, E, F is the average of ten independent experiments.

Figure 2.

A. FIB-SEM was applied to mill MDA-MB-468 cells and to record SEM images at different depths. **B.** SEM image of an individual cell before milling. **C.** Cumulative depth distribution of NP-EGF after incubation times of $t_{\text{inc}} = 15$ min, 30 min, 60 min. **D.** SEM images of identical cellular regions for an exemplary cell recorded at milling depth of 30 nm (left) and 270 nm (right) for cells incubated with NP-EGF for $t_{\text{inc}} = 15$ min (top row), $t_{\text{inc}} = 30$ min (middle row), and $t_{\text{inc}} = 60$ min (bottom row). FIB milling was performed with a rate of approx. 6 nm/s, and the SEM images at depths of 30 nm and 270 nm were obtained after 5 s and 45 s of milling.

Figure 3:

A. Number of NP-EGF per MDA-MB-468 cell, as determined by ICP-MS, as function of input concentration and EGF loading (10, 50, 100, 200 EGF/NP). The NP-EGF were incubated with cells at 37°C for 10 min. The average cell number per ICP-MS run was 20,000. **B.** Same data for HeLa. **C., D.** EGFR phosphorylation determined by flow cytometry as function of NP-EGF input for nanoconjugates with specified EGF loading for MDA-MB-468 and HeLa. The individual data points represent geometric means. The average number of cells per run was 10,000. **E., F.** Phosphorylation signal for NP-EGF with different EGF surface loadings (50, 100, 200 EGF/NP) and free EGF at effective EGF concentrations of 1.5, 3.0, 6.0 nM for MDA-MB-468 and HeLa. **G., H.** Phosphorylation

signal as function of the number of EGF peptides delivered per cell for MDA-MB-468 and HeLa. The data presented in A-H are averages of eight independent experiments.

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Figure 4:

A. Immunoblots of pEGFR (Tyr 1068) and EGFR for (from left to right) blank; 15, 30, 60 pM of NP-EGF (50 EGF/NP); 15, 30, 60 pM of NP-EGF (100 EGF/NP); 15, 30, 60 pM of NP-EGF (200 EGF/NP); and supernatant of 60 pM of NP-EGF (200 EGF/NP) obtained with MDA-MB-468 (top) and HeLa (bottom). Cells were stimulated with NP-EGF and supernatant for 10 min. **B.** Relative phosphorylation for specified experimental conditions for MDA-MB-468. The phosphorylation was determined as ratio of pEGFR and EGFR signals. **C.** Same as in B. but for HeLa. The average number of cells per Western Blot was 20,000.

Figure 5:

A. Confocal image of immunolabelled pEGFR in the dorsal plasma membrane of MDA-MB-468 treated with 3 nM EGF. **B.** Confocal map of immunolabelled pEGFR in the plasma membrane of MDA-MB-468 after treatment with the supernatant of 30 pM NP-EGF (200 EGF/NP). **C.** Top row: Confocal backscattering image of NP-EGF (15pM, 50 EGF/NP) bound to the plasma membrane of an MDA-MB-468 cell (left); confocal scan of the immunolabeled pEGFR in the membrane (right). Bottom Row: merged image (left); computed colocalized pixel map (right). The effective EGF concentration is 0.75 nM. **D.** Same as in C. but with an EGF loading of 200 NP/EGF, corresponding to an effective EGF concentration of 3 nM. **E.** Mander's Overlap Coefficients M1 and M2 for NP-EGF with 200 EGF/NP and 50 EGF/NP in MDA-MB-468 for three input concentrations: 5 pM, 15 pM, 30 pM. **F.** Same as in E. but for HeLa. Size bars are 10 μ m. Data presented in E and F were collected from ten cells.

Figure 6:

Local pEGFR fluorescence intensity *versus* relative NP backscattering signal (normalized by monomer intensity) for **A.** HeLa with 50EGF/NP; **B.** HeLa with 200 EGF/NP; **C.** MDA-MB-468 with 50 EGF/NP; **D.** MDA-MB-468 with 200 EGF/NP. Three different NP-EGF concentration were used 5 pM (blue), 15 pM (red), 30 pM (green). The scattering intensity is normalized by average scattering intensity of an individual NP. **E.** The table summarizes the calculated Pearson Correlation Coefficients (PCC) for fluorescence intensity and scattering signal and associated Student's t-test p-values for the individual conditions. The data in A-D were collected from ten cells per condition.

Figure 7:

A. Confocal map of pEGFR fluorescence (top) and map of peak LSPR wavelength of the same field of view obtained with hyperspectral darkfield imaging (bottom) in a single HeLa cell. NP-EGF with 200 EGF/NP and a concentration of 15 pM were used. The individual pixels are color-coded to identify their peak resonance wavelength in the spectral range 530 nm – 700 nm. Markings a-i indicate positions of colocalizing intensities in both channels. **B.** Histogram of the peak wavelength of cell-bound NP-EGF and NP-EGF bound to glass. **C.** pEGFR fluorescence as function of peak LSPR wavelength obtained from eight individual HeLa cells.