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RGD-decorated conjugated polymer particles as fluorescent biomedical probes prepared by Sonogashira dispersion polymerization†

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Here, we present a facile one-step Sonogashira dispersion polymerization affording monodisperse conjugated polymer particles bearing accessible acetylene moieties on the surface. These acetylene groups are easily functionalized with biological recognition motifs using thiol-yne click chemistry. The resulting functional particles are applied as fluorescent probes for imaging of activated endothelial cells, which take up the particles via receptor mediated endocytosis.

Conjugated polymer particles have broad optical absorption profiles, are tunable in their emission wavelength and display negligible bleaching.¹ Conjugated polymer particles have so far been used in material systems with opto-electronic or photonic applications;^{2–9} however, as they are non-cytotoxic, conjugated polymer particles are gaining importance in cell labelling, *in vivo* imaging, particle tracking and drug or gene delivery.^{10–15} At present, mostly conjugated polymer particles with diameters of around 100 nm are being applied for biomedical studies. These particles are easily prepared from conjugated polymer solutions *via* hot injection and precipitation or by mini-emulsification and solvent evaporation of the dispersed phase.^{10,16–18} Alternatively mini-emulsion droplets can be applied as reaction containers, in which a palladium catalyzed cross-coupling polymerization is performed to obtain the desired particles.¹⁷ However, these systems lack monodispersity as well as simple routes for functionalization with biomolecular recognition motifs to specifically target desired cellular epitopes. A suitable coupling procedure capable of linking a manifold of biological recognition motifs is click-chemistry.¹⁹ As a result, copper catalyzed azide/alkyne click-chemistry has been applied to couple conjugated polymer particles to the surface of cells *via* bio-orthogonal labelling.²⁰

Furthermore, azide functionalized glycans have been applied as recognition epitopes after attachment to an alkyne functionalized conjugated polymer particle, where they bind to glycoproteins on breast carcinoma cells.¹⁵ However, copper residues from the functionalization procedure are problematic during application of the particles when dealing with biological entities and cells. Polydispersity, usually inherent in sets of conjugated polymer particles, is problematic in applications such as particle based cell-labelling and drug- or gene-delivery. All of these applications rely on endocytosis, which is size-dependent and small particles are taken up by cells much faster than larger ones.²¹ When applying polydisperse particles it is impossible to differentiate between unspecific and receptor mediated uptake resulting in non-specific labelling of cells, corrupting the analysis.²² To resolve the problem with polydispersity, we have recently reported a dispersion polymerization protocol for Suzuki–Miyaura cross-coupling, which produces particles in the range of 100 nm up to the micron scale.^{2,4,9,23} The particles are monodisperse with standard deviations of generally below 5%; however, these particles lack functionality on the surface, precluding attachment of biomolecular recognition motifs, and thus obviating the advancement of monodisperse conjugated polymer particles into the fields of fluorescence based cell labelling, analysis and imaging.

Here we report a Sonogashira dispersion polymerization yielding monodisperse particles bearing acetylene groups in the polymer backbone. The acetylene moieties can be addressed for the attachment of biomedical recognition motifs using thiol-containing biomolecules in a copper-free thiol-yne click-reaction.^{24–27} A large variety of biological epitopes is available as either thiol containing cysteine can be easily incorporated or the respective biomolecule is intrinsically carrying this moiety. During thiol-yne click-chemistry the acetylene unit on the surface of the particles is oxidized, yet conjugation and fluorescence are retained by the remaining vinylene bond. The facile one step synthesis followed by the one step thiol-yne click-chemistry allows for versatile preparation of a material toolbox for fluorescent monodisperse particles. The cysteine containing biological recognition motifs can be selected to specifically bind to certain epitopes on cell-surfaces, for example those with

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bio-medical importance, which are only present or over-expressed under pathological conditions, allowing for their detection and 3d imaging.^{28–31}

To obtain monodisperse conjugated polymer particles, we develop a Sonogashira dispersion polymerization in analogy to a previously reported Suzuki–Miyaura dispersion polymerization protocol.⁴ 1-Propanol is employed as a solvent for the monomers and a non-solvent for the resulting polymer. Triton-X45 and poly(vinylpyrrolidone-*co*-vinyl acetate) ($M_w = 50$ kDa) are added to provide stabilization for the emerging particle seeds, which occur after the resulting polymer chains reach their critical molecular weight of solvation. The stabilizers prevent aggregation of the seeds and facilitate growth through condensation of further polymer reaching the critical molecular weight, as is typical in dispersion polymerization (see Fig. 1a, and ESI† for experimentals). The monomers we apply consist of dihalogenated and diacetylene aromatic units and we apply common Sonogashira catalysts to achieve the cross-coupling polymerization. By contrast to Suzuki–Miyaura dispersion polymerization, we apply a more reactive diiodo-monomer and a much softer base, triethylamine, which in combination provide reaction conditions for monodisperse particles (see Fig. 1b and c). The diiodo-species raises the rate of polymerization,³² whereas the soft base does not deactivate the CuI co-catalyst. We use diiodo-diethylfluorene and *m*-diacetylenebenzene as monomers to achieve green fluorescence and we control the size of the particles by varying the monomer concentration, while keeping all other parameters constant (see Fig. 1b). The size can be finely tuned between 140 and 300 nm with dispersities of <5% as determined by image analysis of ~100 particles (see Fig. S2 in the ESI†). At concentrations exceeding 14 mM of monomer we obtain polydisperse particle sets because of secondary nucleation. The particles are stable in relevant media such as deionized water, PBS buffer and cell

culture medium and retain their fluorescence profile also during storage (see Table S1 and Fig. S3 in the ESI†). We test photo-bleaching of our particles by exposing them to 405 nm laser radiation (730 mW) and recording the fluorescence over 10 minutes. The medium has almost no effect on the photo-bleaching effect. Within a few minutes the fluorescence decreases to around 60%, upon which the fluorescence remains relatively stable. We account this to photo-oxidation on the surface, while the particle core does not oxidize and fluorescence is retained. Compared to Pyrromethene 564 doped polystyrene particles our conjugated polymer particles show improved stability and reduced photo-bleaching (see Fig. S4 in the ESI†).

We continue with monodisperse particles smaller than 200 nm in diameter and purify the particles by repeated centrifugation and exchange of the solvent to water or DMSO. For the attachment of a biomolecule we apply a thiol-terminated cyclic (Arg-Gly-Asp-D-Phe-Cys) (cRGD) peptide in 106 molar excess to a dispersion of our fluorene diacetylene phenylene *co*-polymer particles in DMSO.²⁷ For the preparation of a control sample, we functionalize particles with a thiolated oligo ethylene glycol (PEG) chain (see Fig. 1d). The resulting particles experience similar stabilization and surface oxidation, rendering their fluorescence almost identical (see Fig. 2a). After thiol-yne click reaction with either cRGD or PEG, the particles are purified using centrifugation while exchanging the supernatant with milliQ water.

To prove that the surface of our particles is indeed coated with a monolayer of either cRGD or PEG we perform XPS analysis, which clearly show the emergence of nitrogen (N), oxygen (O), and sulphur (S) for cRGD coated particles and O, and S peaks for PEG functionalized particles (see Fig. 2b and Fig. S1, ESI†). This demonstrates successful surface functionalization, as no distinct S and N signals can be seen in the as prepared particles (see Fig. S1, ESI†). The O signal in the as prepared particles will

Fig. 1 (a) Mechanism for the Sonogashira dispersion polymerization. Bifunctional monomers are polymerized in a Pd-catalysed cross-coupling polycondensation in 1-propanol. As the molecular weight of the resulting polymer approaches the critical point of solvation the polymers nucleate. Subsequently formed polymers condensate onto existing nuclei during the growth period, leading to a set of monodisperse particles. (b) By varying the monomer concentrations in Sonogashira dispersion polymerization, the size of the resulting particles can be tuned. Above 14 mM of monomer, the dispersion polymerization affords polydisperse particles. The dashed line is a guide to the eye. The error bars represent the polydispersity as standard deviation from the mean diameter. (c) Scanning electron micrograph of monodisperse particles. The scale bar represents 500 nm. (d) Acetylene units on the surface of the particles can be addressed using thiol-yne click chemistry. Here the particles are functionalized with thiolated PEG (6000 g mol⁻¹) and cRGD.

Fig. 2 (a) Fluorescence spectra of PEG (black line) and cRGD (blue line) functionalized particles excited at 350 nm, showing almost identical fluorescence profiles with a maximum at 520 nm. (b) XPS measurement on cRGD functionalized particles shows surface modification. (c) HUVECs incubated with control particles. Only few particles are visible after 6 hours. (d) cRGD functionalized particles incubated with HUVECs for 6 hours. Most particles are either adhering or have been taken up by the cells. Cell nuclei in (c) and (d) are stained with DAPI. The scale bars represent 50 μm .

originate from residual Triton-X45 stabilizer on the particle surface. Both surface functionalities can be attached following the same and facile protocol proving the flexibility and potential as a material toolbox. To test whether the cRGD surface functionalization aids attachment and endocytosis by cells expressing $\alpha\text{V}\beta 3$ integrins, we incubate human umbilical vein endothelial cells (HUVECs) with cRGD functionalized particles and PEGylated control particles without a recognition motif. HUVECs represent activated endothelial cells, which are overexpressing $\alpha\text{V}\beta 3$ integrins. This activated state is typically also observed for endothelial cells in tumor blood vessels and HUVECs can be considered a model system for the tumor endothelium. After 4 to 6 hours we can clearly see a difference in labelling of the cRGD functionalized *versus* the control particles. Whereas the cRGD

particles are close to the DAPI stained nuclei of HUVECs, there are much less control particles close to the nuclei (see Fig. 2c and d). This clearly demonstrates the cRGD surface activity and recognition of membrane bound integrins compared to the particles without the recognition motif (see Fig. 2c and d). To determine the cytotoxicity of the particle system, we incubate particles with HUVECs for 24 hours and test the cell viability using Trypan Blue staining. We could not observe cytotoxicity, neither for surface functionalized particles nor for a sample without surface functionality (see Fig. 3a). This demonstrates that our particles are generally biocompatible and non-cytotoxic. To further investigate the binding of cRGD functionalized particles to $\alpha\text{V}\beta 3$ integrins at the cell membrane, we perform binding studies in a flow chamber setup, where the particles are flown over adherent HUVECs and binding of the particles to the cells is monitored. We compare binding of the PEGylated control particles *versus* cRGD-functionalized particles. For the control conjugated polymer particles we only see very low binding. However, for cRGD particles we obtain approximately a threefold increase in particle binding to HUVECs (see Fig. 3b). When we perform a competition experiment by adding an excess of non-particle bound cRGD molecules, we observe reduced binding similar to the non-binding PEG-functionalized particles, demonstrating specific binding of the cRGD particles to $\alpha\text{V}\beta 3$ integrin on the cell surface of the HUVECs. Note the relatively large standard deviations, which originate from heterogeneity in the HUVEC binding assays, where we analyzed >2000 cells in several flow-chamber experiments (see Fig. 3b). To test the reliability of our binding study, we perform one-way ANOVA combined with Bonferroni correction and obtain significance in all cases ($p < 0.05$, as indicated by the asterisks in Fig. 3b). We can hence conclude that cRGD to $\alpha\text{V}\beta 3$ integrin recognition is indeed part of the particle uptake mechanism. When scanning through cells in 3d (from bottom $z = 0$ to top $z = 7$ in Fig. 3c) we can see that the cRGD functionalized particles are actually inside of the cells after an incubation time of 6 hours.²²

Endocytosis mediated by $\alpha\text{V}\beta 3$ integrin binding is accelerated, as we observe much less uptake of control particles at the same

Fig. 3 (a) Cytotoxicity test: HUVECs are incubated for 24 h with either no particles, cRGD functionalized conjugated particles and non-functionalized particles (particle concentration 250 $\mu\text{g mL}^{-1}$). (b) PEG and cRGD particles are flown over adherent HUVECs. The average number of particles bound per cell is plotted on the y-axis. As a competition experiment, an excess of free cRGD is added to the cRGD functionalized particle dispersion. Replacement of the particles with free RGD proves binding of cRGD to $\alpha\text{V}\beta 3$ integrins on the HUVEC cell membrane. (c) Confocal microscopy z-scan of two HUVECs from the bottom ($z = 0$) to the top ($z = 7$) of the cell. Cell nuclei are stained with DAPI. The scale bars represent 5 μm .

point in time (cf. Fig. 2c, d and 3b, c). Previous studies describe that HUVECs internalize particles ≤ 200 nm much better than larger particles.²¹ We observe that the conjugated polymer particles of 200 nm in diameter get taken up much faster than particles with a diameter of 300 nm. When comparing the latter with cRGD-functionalization to non-functionalized control particles of the same size, we can only observe a clear difference in uptake after 24 hours. This difference in uptake is already substantial after 4–6 hours in case of particles with a diameter of 200 nm. This demonstrates how important monodispersity is to obtain representative results for *in vitro* integrin binding and cellular uptake and the necessity to work with particles ≤ 200 nm to enable early discrimination between cells with integrin over-expression and other cells.

In the future, to drive this field of conjugated polymer diagnostics further towards *in-vivo* application, near-IR emitters will have to be developed to allow excitation and fluorescence detection in the tissue transparency window. The results presented here showcase an easy approach to produce highly selective particle systems, which bind selectively to $\alpha V\beta 3$ integrins on activated endothelial cells under flow and static cell culture conditions. The particles can easily be functionalized with different recognition motifs, demonstrating the potential and versatility in this type of particles.

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