



Universiteit
Leiden
The Netherlands

Coiled-coil biomaterials for biological applications

Shen, M.

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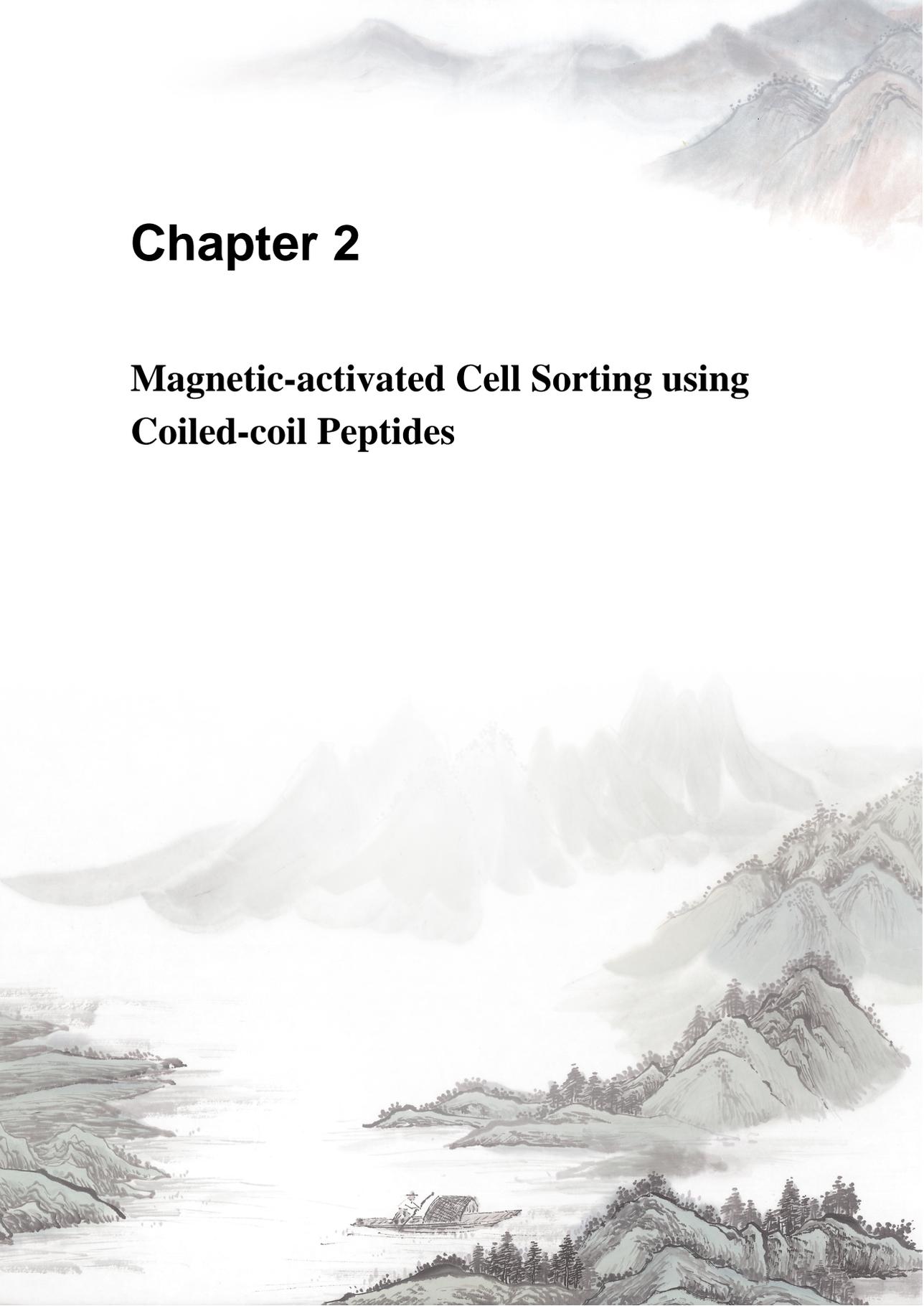
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Chapter 2

Magnetic-activated Cell Sorting using Coiled-coil Peptides



ABSTRACT

Magnetic-activated cell sorting (MACS) is an affinity-based technique used to separate cells according to the presence of specific markers. Current MACS systems generally require an antigen to be expressed at the cell surface; these antigen-presenting cells subsequently interact with antibody-labelled magnetic particles, facilitating separation. Here, we present an alternative MACS method based on coiled-coil peptide interactions. We demonstrate that HeLa, CHO and NIH3T3 cells can either incorporate a lipid-modified coiled-coil-forming peptide into their membrane, or that the cells can be transfected with a plasmid containing a gene encoding a coiled-coil-forming peptide. Iron oxide particles are functionalized with the complementary peptide and, upon incubation with the cells, labelled cells are facily separated from non-labeled populations. In addition, the resulting cells and particles can be treated with trypsin to facilitate detachment of the cells from the particles. Therefore, our new MACS method promotes efficient cell sorting of different cell lines, without the need for antigen presentation, and enables simple detachment of the magnetic particles from cells after the sorting process. Such a system can be applied to rapidly developing, sensitive, research areas, such as the separation of genetically modified cells from their unmodified counterparts.

INTRODUCTION

The sorting of specific cells from complex mixtures is necessary for a variety of applications, ranging from cancer research,¹⁻⁴ to assisted reproduction,⁵ and cell-based therapies,⁶⁻⁷ in addition to the selection of genetically modified cells.⁸⁻⁹ Two of the primary affinity-based techniques used for cell sorting are fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS).¹⁰⁻¹¹ Whilst FACS has several advantages: analysis is rapid and multiple parameters can be simultaneously analyzed, the disadvantages include the fact that expensive, specialist equipment is needed and cells must be modified to display a fluorescent moiety. MACS can circumvent these disadvantages: no specialist equipment is required and no fluorescent labels are needed. Instead, MACS employs magnetic particles that can be functionalized to enable binding to a subset of cells in a mixture, facilitating separation.¹²⁻¹⁵ Usually, the particles are functionalized with an antibody, which is specific for antigens expressed on the surface of cells of interest. The beads and the cells are incubated and subsequently placed in a magnetic field. Cells that do not express the antigen of interest are not retained in the magnetic field, whereas cells that do display the antigen of interest bind to the beads and are retained. Once the magnetic field is removed, the cells of interest can be eluted. However, MACS does have disadvantages: functionalization of the iron oxide particles (IOPs) with antibodies is not trivial, and such antibodies are typically expensive. Indeed, one study highlighted that, when MACS was used to select for induced pluripotent stem cells from a cell mixture, the antibody comprised 65% of the purification cost.¹⁶ MACS systems can also suffer from low cell purity after separation due to non-specific binding between the cells and the functionalized magnetic particles.¹⁷⁻¹⁸ In addition, it is not trivial to separate the cells from the magnetic particles, which can lead to adverse effects. For example, magnetic particles can influence the phenotype and function of some cells,¹⁹⁻²⁰ in addition to affecting cell viability.²¹⁻²²

Therefore, there is a need to design and synthesize functionalized magnetic particles that possess a high specificity for the cells of interest and are facile to dissociate from the cells after separation. Such a system would benefit multiple areas of cell biology and medicine, for example facilitating the separation, and subsequent enrichment, of genetically modified cells.

Coiled coils are a protein-folding motif comprising two or more alpha-helices that interact to form a left-handed supercoil.²³ Different oligomer states, orientations of

helices, and both homomeric and heterodimeric assemblies are possible.²⁴ Moreover, rules exist for the programmable design of such structures,²⁵⁻²⁶ which has enabled synthetic coiled-coil systems to be designed and employed in a variety of applications.²⁷⁻³⁰ We were motivated to determine whether coiled-coil peptides could be used to separate cells in a MACS-based approach. Therefore, we designed a MACS system based on interactions between magnetic beads and cells that were functionalized with complementary coiled-coil forming peptides, (Figure 1). We employed a heteromeric coiled-coil system, dubbed E₃/K₃.³¹ Nanometer-sized iron oxide magnetic particles (IOPs) were coated with dextran-divinyl sulfone (dextran-DVS) and subsequently functionalized with E₃ to yield IOPs-E₃. HeLa, CHO and NIH3T3 cells were incubated with a lipidated K₃ derivative, (CPK), which is known to spontaneously incorporate into the cell membrane.³² When the cells and the functionalized IOPs were mixed, cells that displayed K₃ on their surface bound to the IOPs-E₃ via the formation of a coiled-coil. Subsequent application of an external magnetic field facilitated isolation of these cells with high efficiency and specificity, (Figure 1A). Another advantage of this system is that the coiled-coil peptides could be degraded by trypsin, which made the dissociation of the cells from the IOPs facile and efficient. We subsequently demonstrate that HeLa, NIH3T3, and CHO cells can be transfected with a plasmid containing K₃. These cells express K₃ on their membrane and we show that these cells could be separated, (Figure 1B), and subsequently enriched, from non-transfected cells. These results demonstrate that our coiled-coil based MACS system can facilitate cell separation, and subsequent enrichment of transfected cells, with high specificity and efficiency.

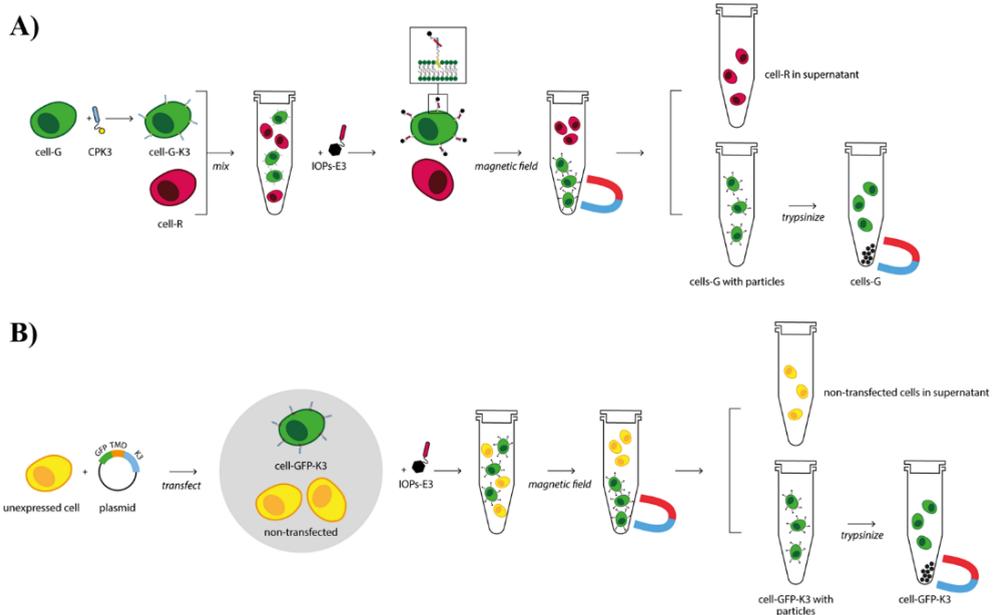


Figure 1. Coiled-coil-based MACS. Cells are either functionalized with a coiled-coil forming peptide (A) or transfected with a K₃-containing plasmid (B); low transfection rates mean not all cells express the K₃ peptide. IOPs bearing the complementary peptide are added and separation is facilitated by coiled-coil formation and application of a magnetic field. Post-separation, cells are separated from the IOPs *via* trypsinization.

RESULTS AND DISCUSSION

Design and synthesis of peptide-functionalized IOPs

Functionalized magnetic particles suitable for cell sorting need to possess several properties including: specificity for the cells of interest; high binding and separation efficiency, and; effective dissociation. To fulfil these criteria a coiled-coil-functionalized IOP system was designed. Two complementary peptides are known as K₃, (KIAALKE)₃, and E₃, (EIAALEK)₃, named for the prevalence of lysine and glutamic acid residues in their respective sequences, were employed.³¹ These two peptides interact to form a heterodimeric coiled coil with a micromolar dissociation constant. This tight binding enables peptide-functionalized IOPs to bind to the complementary peptide with high efficiency, (Figure 2A).

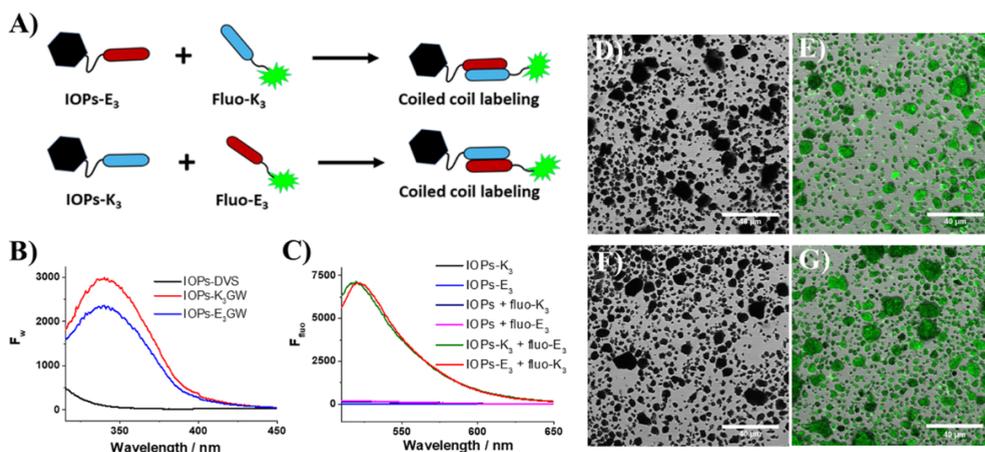


Figure 2. Coiled-coil-functionalized magnetic particles. A) Schematic of fluorescent labelling of the IOPs: coiled-coil functionalized IOPs are incubated with the complementary fluorescent peptide. B) Tryptophan fluorescence spectrum of functionalized IOPs, indicating attachment of the peptides to the particles. C) Fluorescein fluorescence spectrum of fluorescently-labeled IOPs; a fluorescein spectrum is only observed when the IOPs are labeled with the complementary peptide. D-G) Confocal microscopy images of: IOPs-DVS treated with fluo-K₃ (D); IOPs-E₃ treated with fluo-K₃ (E); IOPs-DVS treated with fluo-E₃ (F), and; IOPs-K₃ treated with fluo-E₃ (G). Scale bar: 40 μm.

IOPs need to be coated to reduce non-specific interactions with cells and to facilitate functionalization with a moiety specific to the cells of interest. In this study, IOPs were synthesized and coated with a dextran-divinyl sulfone (Dex-DVS) polymer in a one-pot reaction. The DVS functionality facilitates labelling of the IOPs with any compound containing a free sulfhydryl group *via* a Michael addition. An added advantage is that the number of DVS groups can be adjusted by synthesizing Dex-DVS with differing degrees of substitution, allowing for control over the number of functional groups displayed on the surface of the IOPs.

Conjugation of the coiled coil-forming peptides to the IOPs was facilitated by modifying the peptides to include a free sulfhydryl group. To this end, Ac-E₃GW-PEG₄-Cys and Ac-K₃GW-PEG₄-Cys were designed, these peptides incorporate a cysteine (Cys, C) at their C-terminus. A polyethylene glycol (PEG) spacer was included between the cysteine and the rest of the peptide sequence to minimize potential steric hindrance, which may impact coiled-coil formation. A tryptophan (Trp, W) was included to facilitate detection and quantification of the peptide.

To demonstrate the peptide-functionalized IOPs were successfully synthesized, fluorescence spectroscopy was employed. IOPs functionalized with the coiled-coil-forming peptides exhibit a fluorescence spectrum corresponding to that of Trp, (Figure 2B), which indicates the peptides were successfully conjugated to the IOPs.

Coiled-coil formation was subsequently confirmed using a fluorescence labelling assay. The fluorescein conjugated peptides, fluo-K₃ and fluo-E₃, were mixed with either non-functionalized IOPs or IOPs bearing the complementary peptide. Figure 2C shows that a fluorescein fluorescence spectrum is only observed when the peptides are mixed with IOPs functionalized with the complementary peptides. This indicates coiled-coil formation and demonstrates that no non-specific binding between fluo-E₃ or fluo-K₃ and non-functionalized IOPs occurs. The results were verified by confocal microscopy imaging, (Figure 2D-G). The unmodified IOPs did not exhibit fluorescence after incubation with fluo-K₃ or fluo-E₃, (Figure 2D&F), whereas peptide-modified IOPs have a green fluorescent surface after labelling with the complementary peptide, *i.e.* IOPs-E₃ + fluo-K₃, (Figure 2E) or IOPs-K₃ + fluo-E₃, (Figure 2G).

MACS for lipopeptide-decorated cells

We have previously employed the lipopeptides CPK and CPE to facilitate membrane fusion.³³⁻³⁴ These lipopeptides comprise the K and E peptides, with a PEG spacer that connects the peptide to a cholesterol anchor. This anchor enables the insertion of the lipopeptide into the lipid bilayer of a cell membrane. By functionalizing specific cells with these lipopeptides and adding IOPs functionalized with the complementary coiled-coil forming peptide, cells can be separated from others in a mixture.

To confirm that the lipopeptides synthesized for this study are capable of inserting into the cell membrane and subsequently forming a coiled-coil, a cell membrane labelling assay was performed. The fluo-K₃ or fluo-E₃ peptides were added to cells decorated with the complementary lipopeptide. Using confocal microscopy, it was determined that the lipopeptide-decorated cells exhibited a fluorescently-labelled membrane, while no fluorescent labelling was observed on non-decorated cell membranes, (Figure S1).

After it was confirmed that coiled-coil forming peptides could be used to functionalize IOPs and cell membranes, and that coiled-coil formation occurred, a proof-of-principle coiled-coil-mediated MACS experiment was designed. Separate

HeLa cell populations were stained with either CellTracker™ Green or CellTracker™ Red. Green cells were incubated with CPK₃ for one hour, before being mixed with the same number of red cells. IOPs-E₃ were subsequently added to the cell mixture. It was anticipated that the IOPs-E₃ would selectively bind to the CPK₃ decorated green cells via coiled-coil formation. Through the application of an external magnetic field, cells attached to the magnetic particles could be isolated. The IOP-attached cells could subsequently be treated with trypsin to dissociate the magnetic particles before an external magnetic field could again be used to separate the IOPs from the detached HeLa cells.

Confocal imaging was performed directly after MACS; the results are shown in Figure 3. Before MACS, the cell population contained CPK₃ decorated green cells and undecorated red cells (Figure 3A). After MACS, only red cells were found in the supernatant (Figure 3B) and cells attached to the IOPs were almost exclusively green (Figure 3C). This demonstrates that the K₃/E₃ coiled-coil-based MACS system can be used to efficiently separate cell populations. To demonstrate that the cells can be cleaved from the IOPs, they were incubated with trypsin and then separated from the IOPs using an external magnetic field. Figure 3D shows the image of the cells after dissociation: most of the cells exhibit green fluorescence. The cell-IOPs dissociation is efficient: no cells were observed to remain attached to the IOPs (Figure 3E). After MACS, the cells were allowed to grow for 24 h before imaging again to demonstrate that both the cells from the supernatant, (Figure 3F) and the cells detached from the IOPs, (Figure 3G) remain viable. The same study was then performed using CHO and NIH3T3 cells to illustrate the broad applicability of this MACS system, (Figures S2-S3).

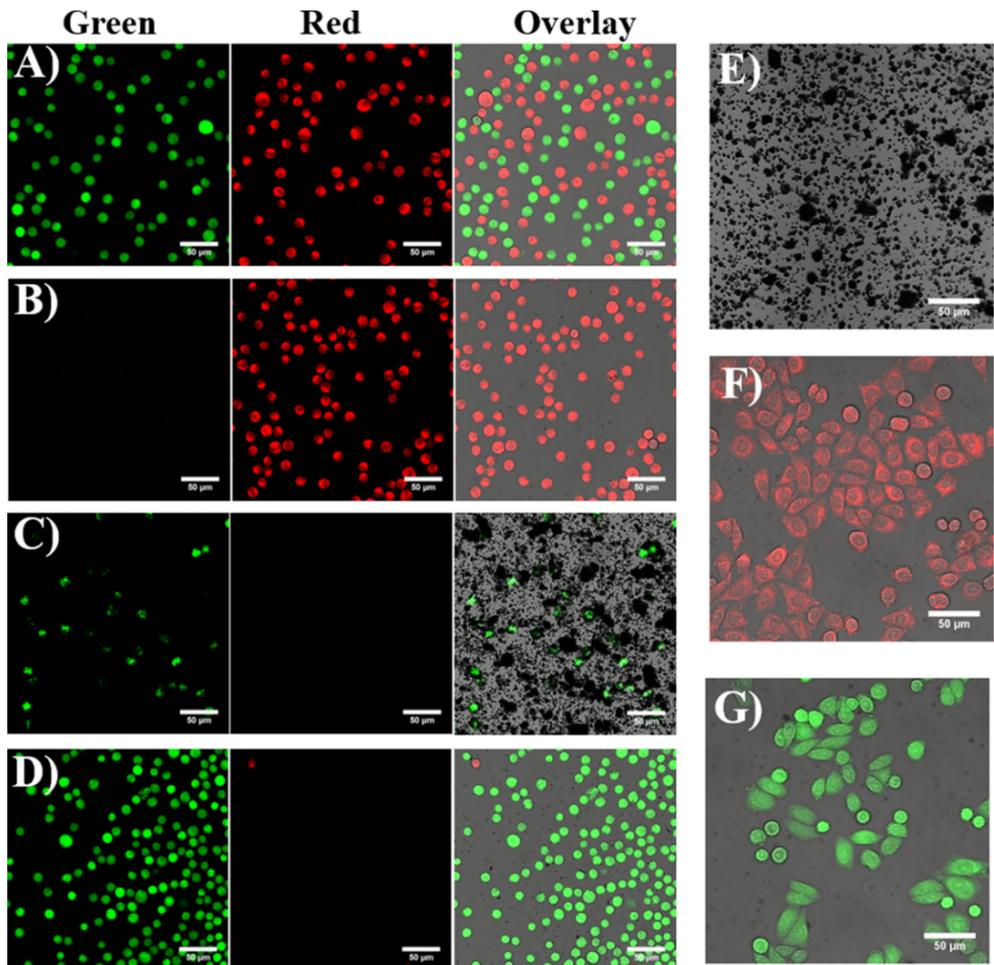


Figure 3. Coiled-coil-facilitated MACS of HeLa cells. A) CPK₃ modified HeLa cells stained with CellTracker™ green mixed with non-functionalized HeLa cells stained with CellTracker™ red; B) cells in the supernatant after MACS; C) IOP-attached cells after MACS; D) cells detached from the IOPs after trypsinization; E) no visible cells remain attached to the IOPs after trypsinization; cells from the supernatant (F) and those detached from IOPs (G) show signs of recovery and growth after 24 hours. Scale bar: 50 µm.

Whilst confocal imaging provides a qualitative impression of the efficiency of this system, quantification of cell separation is desirable. Therefore, flow cytometry was employed for all three cell lines. Before MACS, the flow cytometry data shows the cells are mixed in an approximately 1:1 ratio, as designed, Table 1. After one round of MACS, the cells were demonstrated to be highly efficiently separated as, for all three cells lines tested, more than 99% of the cells in the

supernatant were red cells and more than 94% of cells detached from the IOPs were green cells (Table 1, Figures S4-S6).

Table 1. FACS quantification of cell populations before and after MACS.

Cell line	Before MACS (%)		After MACS: Supernatant (%)		After MACS: IOP detached cells (%)	
	Red	Green	Red	Green	Red	Green
Hela	51.5	47.2	99.2 (0.2) ^a	0.5 (0.2)	4.9 (1.5)	94.8 (1.3)
CHO	44.5	55	99.7 (0.0)	0.3 (0.0)	4.3 (0.1)	95.7 (0.1)
NIH3T3	48.5	51.5	100 (0.0)	0 (0.0)	0.7 (0.2)	99.3 (0.2)

^a Error is calculated as the standard deviation from the average of at least two independent measurements.

Alternative IOP Functionalization

Cell membranes are usually negatively charged. For this reason, IOPs were functionalized with E₃ and not K₃ as it was hypothesized that IOPs-K₃ could non-specifically interact with the negatively charged cell membrane. To verify this hypothesis, an experiment was performed using Hela cells modified with CPE₃ and IOPs functionalized with K₃, (Figure S7). The CPE₃ decorated green cells were mixed with red cells in a 1:1 ratio (Figure S7A). The cell mixture was incubated with IOPs-K₃ and after MACS, only red cells were found in the supernatant (Figure S7B). However, a mixture of red and green cells were found to be attached to the IOPs, (Figures S7C); this is more evident after trypsinization, (Figure S7D). In addition, FACS analysis was performed and this revealed that the cells detached from the IOPs have almost equal populations of green and red cells (Figure S7E & S8).

Combined, these experiments reveal that coiled-coil-forming peptide-functionalized IOPs can be used to efficiently sort cells bearing the complementary coiled-coil-forming peptide from a mixture. This system can be applied to a variety of different cell lines. The data also indicates that the charge of the peptide which is conjugated to the IOPs makes a significant difference to the efficiency of the cell sorting process.

MACS with GFP-K3 expressed cells

For the proof-of-principle experiment, cells have to be manually decorated with K_3 before cell sorting; this process limits the potential applications of the MACS system. To fully benefit from using coiled coils as a cell-surface marker, cells that express K_3 on their membranes were employed, (Figure 1B): such a system has been used previously in our lab.³⁴ The K_3 peptide was fused to a signal sequence and a transmembrane domain (TMD) from the platelet-derived growth factor receptor beta (PDGFRB). The presence of this signaling sequence and the TMD ensures that the K_3 peptide is transported to, and anchored in, the cell membrane. In addition, GFP was included to act as a fluorescent marker to illustrate the expression of the K_3 -TMD-GFP construct after transfection. GFP can also be used as a fluorescent label for FACS quantification before and after MACS.

After cell transfection and two weeks of antibiotic selection, approximately 10% of all transfected cells successfully expressed the K_3 -TMD-GFP construct. A cell-labelling experiment was subsequently performed using a Cy5-conjugated E_3 peptide to confirm that the K_3 peptide was successfully expressed on the cell membrane, (Figure 4A).

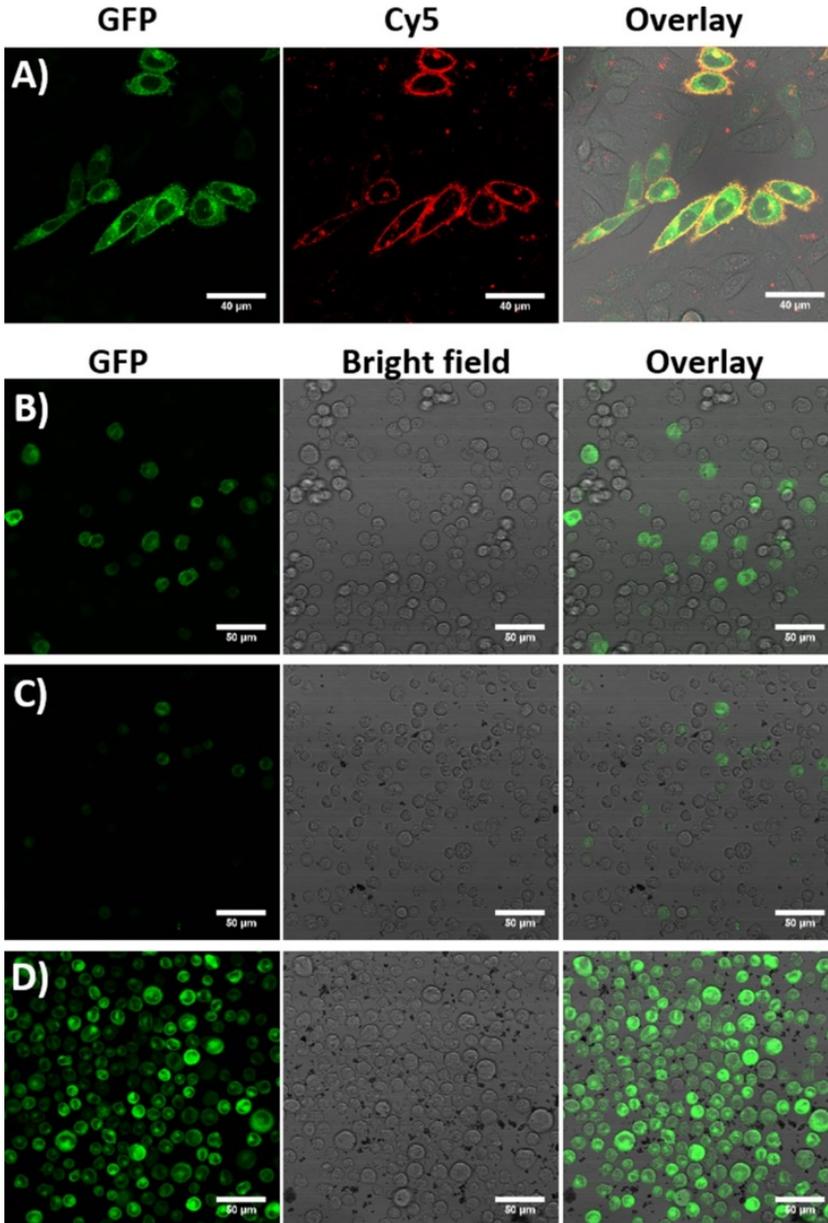


Figure 4. HeLa cell membrane labelling and cell sorting. A) HeLa cells were transfected with a plasmid containing the K_3 -TMD-GFP gene. The transfected cells were subsequently incubated with Cy5- E_3 to demonstrate successful expression of K_3 at the cell surface. Green channel: GFP, Red channel: Cy5. Scale bar: 40 μ m. Transfected cells were then subjected to MACS: (B) before MACS; (C) cells from the supernatant after MACS; (D) cells detached from IOPs after MACS. Green channel: GFP. Scale bar: 50 μ m.

As the K_3 peptide was indeed expressed on the cell membrane, it was possible to use it as a selection marker for cell sorting. As GFP is co-expressed in the K_3 -expressing cells, confocal microscopy can be employed to track the cell sorting process. Before MACS, a low percentage of green cells were found in cell mixture due to the low transfection efficiency (Figure 4B). IOPs- E_3 were added to the cells and an external magnetic field was applied. After MACS, few green cells were observed in the supernatant, (Figure 4C), whereas cells retained in the magnetic field and then detached from the IOPs were GFP-expressing cells, (Figure 4D). This shows that the coiled-coil peptide-based MACS system is capable of selective separation of K_3 -expressing cells from non-transfected cells. Although some cells that expressed GFP were found in the supernatant, the fluorescence level was low, indicating that the expression level of K_3 could also affect the separation. The cells were subsequently cultured for two days: Figure 5A shows that the GFP positive cells could be enriched to a high level after coiled-coil based MACS. FACS quantification showed only 17.4% of GFP positive cells in the transfected cell mixture before MACS, (Table 2, and Figure S9), but after MACS selection, 94.3% of GFP positive cells were obtained. These results confirm that GFP- K_3 expressed cells can be effectively enriched by this MACS system.

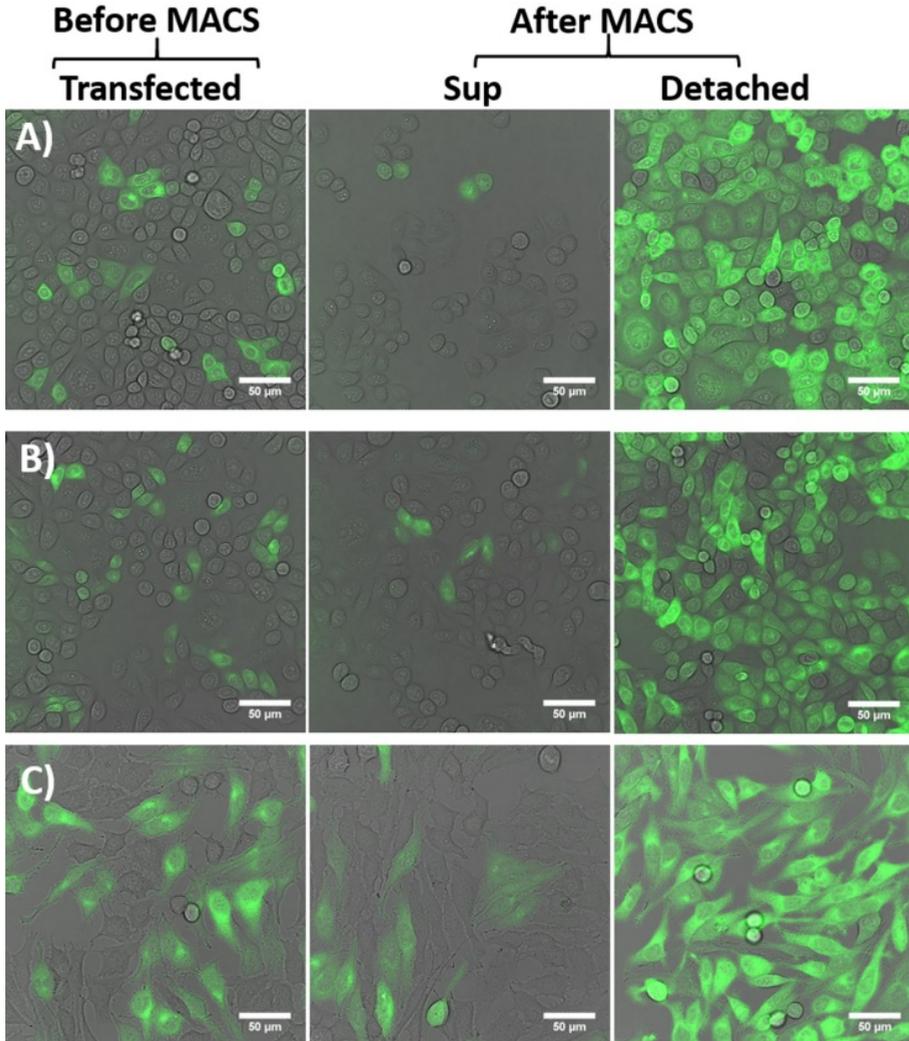


Figure 5. Imaging of K₃-TMD-GFP cells before and after MACS. A) Hela-K₃ cells, B) CHO-K₃ cells, C) NIH3T3-K₃ cells. The left column shows transfected cells before MACS, the middle column shows cells in the supernatant after MACS and cells detached from IOPs after MACS are on the right. All images are an overlay of bright field and fluorescence microscopy images. Scale bar: 50 μm.

To demonstrate the wider applicability of the transfected MACS system for cell sorting and enrichment, CHO and NIH3T3 cell lines were again employed, (Figure 5B & C). Cells were analyzed using FACS after separation, (Figures S10 & 11). For CHO cells, an enrichment from 14.8% to 76% of GFP-expressing cells could be obtained after MACS. This system is even more successful for NIH3T3 cells;

the FACS data shows that 24.2% of GFP-K₃ expressing NIH3T3 cells could be enriched to 95.6% after MACS.

Table 2. FACS quantification of GFP positive cells before and after MACS.

Cell line	Before MACS (%)	After MACS (%)	
		Supernatant	Detached
Hela	17.4	11.7 (0.6) ^a	94.3 (2.2)
CHO	14.8	12.2 (8.1)	76.0 (2.5)
NIH3T3	24.2	19.8 (0.1)	95.6 (0.4)

^a Errors are calculated as the standard deviation from the average of at least two independent measurements.

Both the qualitative and quantitative data show that the GFP-K₃ expressing cells from different cell lines can be efficiently isolated from a cell mixture using IOPs-E₃. These results demonstrate that the coiled-coil-mediated MACS system can be applied to enrich cell populations. For this study, GFP was co-expressed with K₃ to facilitate both imaging and quantification by FACS. However, MACS itself doesn't require the cells to express GFP or indeed any fluorophore, which is beneficial for studies where fluorescent proteins are detrimental and therefore FACS is not possible. Additionally, the GFP could be replaced by any protein of interest and a single round of coiled-coil-mediated MACS will separate the cells expressing this protein with high specificity and efficiency.

CONCLUSIONS

In this study, we have taken advantage of coiled-coil peptide interactions and designed a new MACS system based on this non-covalent interaction. This MACS system facilitates efficient, facile cell sorting. A particularly attractive feature of this system is that the isolated cells can be easily dissociated from the IOPs by trypsinization, meaning the magnetic particles do not remain attached to the cells. This system may not be suitable for all cell types, particularly those isolated from tissues, due to the need to incorporate an extrinsic selection marker. However, the fact that the K₃ peptide can be employed as a selection marker for transfected cells means that this approach has the potential to become an alternative method for transfected cell selection, eliminating the need for FACS or antibiotics. Moreover, the plasmid can be modified to include any gene of interest, either as a fusion with

K₃ or by including cleavage sites. Such a system could therefore have applications in a wide range of biomedical areas that require cell separation.

EXPERIMENTAL SECTION

Chemicals

All chemicals were purchased from Sigma unless otherwise stated. Amino acids and HCTU were purchased from Novabiochem. Tentagel HL RAM resin was purchased from Iris Biotech GmbH. Piperidine, trifluoroacetic acid, acetic anhydride and all other solvents were purchased from Biosolve. Oxyma pure was purchased from Carl Roth GmbH. Dextran 70 was supplied by Pharmacosmos. CellTracker™ red and green, lipofectamine 3000 and 2 KDa MWCO dialysis membrane were purchased from Thermo Fisher. Confocal Chambered Coverslips (μ -Slide 8 Well) were purchased from Ibidi. All cell culture supplies were purchased from Starstedt. Carbon/Formvar grids for transmission electron microscopy were purchased from Agar Scientific.

Peptide synthesis

For the synthesis of CPE₃ and CPK₃, peptides K₃ (KIAALKE)₃ and E₃ (EIAALEK)₃ were synthesized using Fmoc chemistry on a CEM Liberty Blue microwave-assisted peptide synthesizer. DIC was used as the activator and Oxyma as the activator base. Fmoc deprotection was performed with 20% piperidine in DMF. The peptide was synthesized on a Tentagel HL RAM resin (0.39 mmol/g). Once synthesis of the peptide was complete, two eqv. N₃-PEG₄-COOH (synthesized as described elsewhere),³⁵ were manually coupled to the peptides on resin, using three eqv. HCTU and five eqv. DIPEA in DMF. The resin was washed with DMF after three hours. Five eqv. trimethylphosphine (1M in toluene) in a dioxane:H₂O (6:1) mixture were added to the resin to reduce the azide. After three hours, the resin was washed with dioxane followed by DMF before cholesteryl hemisuccinate (three eqv.) was coupled to the N-terminus of the PEG linker using three eqv. HCTU and five eqv. DIPEA in DMF. After overnight coupling, the resin was washed with DMF followed by DCM and the resulting CPK₃ or CPE₃ construct was cleaved from the resin by adding 5 ml of a TFA:triisopropylsilane (97.5:2.5%) mixture and shaking for 45 mins. The crude peptide was precipitated by pouring into 45 mL cold diethyl ether and isolated by centrifugation. The

peptide pellet was dissolved in 20 mL H₂O with 10% acetonitrile and freeze-dried to yield a white powder.

The fluo-E₃ and fluo-K₃ peptides were synthesized in a similar manner. Two additional glycine residues were coupled to the N-terminus of the K₃ and E₃ peptides. 5(6)-carboxyfluorescein (three eqv.) was subsequently manually coupled to the peptides using four eqv. HCTU and six eqv. DIPEA in DMF. After an overnight reaction, the resin was washed using DMF and the fluorophore-labelled peptides, flu-K₃ and flu-E₃, were cleaved from the resin using 5 mL TFA:triisopropylsilane:H₂O (95%:2.5%:2.5%) for one hour. The peptide was precipitated into diethyl ether, centrifuged, redissolved in H₂O and MeCN and freeze-dried.

For the Ac-E₃GW-PEG₄-Cys and Ac-K₃GW-PEG₄-Cys peptides, cysteine was coupled to Tentagel HL RAM resin before N₃-PEG₄-COOH was coupled to the cysteine using the procedure described above. After three hours, the resin was washed and the azide was reduced. The resin was transferred to the peptide synthesizer and either E₃: (EIAALEK)₃GW or K₃: (KIAALKE)₃GW was synthesized. Upon completion of the synthesis, the resin was manually acetylated by adding 3 mL of an acetic anhydride:pyridine:DMF (5%:6%:89%) solution. After one hour, the resin was washed with DMF and DCM, before the peptide was cleaved from the resin by adding 5 mL TFA:TIPS:Et₃N:H₂O (92.5:2.5%:2.5%:2.5%). After two hours, the peptide was precipitated into diethyl ether, centrifuged, redissolved and freeze-dried.

Cy5-E₃ was synthesized for labelling GFP-K₃ expressing cells. E₃ was synthesized using the method described above. After the synthesis, three eqv. 4-pentynoic acid, four eqv. HCTU and six eqv. DIPEA in 2 mL DMF were added to the E₃ peptide, on the resin, to facilitate coupling of an alkyne to the N-terminus. After one hour, the resin was washed (DMF followed by DCM) and the peptide was cleaved. The Alkyne-E₃ peptide was purified using HPLC (see below) before coupling to Azide-Cy5. This coupling was performed by dissolving 1.55 mg (9.7 nmol) CuSO₄ in 1 mL H₂O. 19 mg (97 nmol) of L-ascorbic acid was subsequently added and the color of the solution became brown, before turning light yellow. 21 mg (48.5 nmol) Tris(3-hydroxypropyltriazolylmethyl)amine was dissolved in 150 μ L DMSO and added to solution, which was stirred for 3 min at 500 rpm before 1 mg (0.97 nmol) Azide-Cy5 in 500 μ L H₂O was added, followed by 11.5 mg (4.85 nmol) Alkyne-E₃ in 500 μ L H₂O. The reaction was stirred for 2 hours before being dialyzed

overnight using a 2 KDa MWCO membrane. The resulting Cy5-E3 peptide was purified using HPLC.

Peptide Purification

Peptide and lipopeptide purification was performed using reversed-phase HPLC on a Shimadzu system with two LC-8A pumps and an SPD-20A UV-Vis detector.

Lipopeptide (CPK₃ and CPE₃) purification was performed with a Vydac C4 column (22 mm diameter, 250 mm length, 10 μ m particle size). A linear gradient from 20% to 80% acetonitrile (with 0.1% TFA) in water (with 0.1% TFA) was performed over 36 mins, with a flow rate of 12 mL/min.

Peptides Ac-K₃GW-PEG₄-Cys, Ac-K₃GW-PEG₄-Cys, fluo-E₃, fluo-K₃, E₃, and Cy5-E₃ were purified using a Kinetix Evo C18 column (21.2 mm diameter, 150 mm length, 5 μ m particle size). A linear gradient from 20% to 55% acetonitrile (with 0.1% TFA) and water (0.1% of TFA) was used for the HPLC method: the running time was 28 mins, and the flow rate was 12 mL/min.

After purification, the collected fractions of all lipopeptides and peptides were assessed using LC-MS (Figures S12-S15, Table S1). Fractions that were deemed to be >95% pure were combined and lyophilized.

Modification of Dextran with DVS

The synthesis of Dextran-DVS was performed according to a previously reported protocol.³⁶ Briefly, 10 g (61.7 mmol) of Dextran 70 was dissolved in 300 mL of a 0.1 M NaOH solution in an ice bath. Afterwards, 23 mL (229 mmol) divinyl sulfone was added under vigorous stirring (1000 rpm). The reaction was left for 65 s before 5 mL of 6 M HCl was added to acidify the solution to pH 5.0. Dextran-DVS precipitation was achieved after 300 mL cold isopropanol was added. The gel-like precipitate was dissolved in H₂O and dialyzed using 2 KDa MWCO dialysis tubing; the dialysis solution was changed every 12 hours, and the dialysis was left for three days. The resulting dextran-DVS solution was concentrated to a final volume of 100 mL by overnight exposure to an N₂ stream. The solution was dried and 7.5 g (yield = 74%) of lyophilized powder was obtained. A schematic detailing the entire synthesis process is shown in Figure S16.

The degree of substitution (DS) of dextran can be defined as the number of vinyl sulfone groups per 100 glucopyranose residues. The calculated DS of dextran

which has been used for coating IOPs in this work is 4.6 and the calculation is based on ^1H NMR (Figure S17). The DS can be controlled by altering the reaction time, therefore it is possible to obtain dextran-DVS with a higher DS. However, the solubility of DVS-modified dextran was found to negatively correlate with the DS, therefore it was determined that dextran-DVS with a DS of 4.6 offered the optimal balance between solubility and functional group density.

Dextran-DVS coated magnetic IOPs synthesis (IOPs-DVS)

0.88 g (5.4 mmol) FeCl_3 and 0.55 g (2.7 mmol) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were dissolved in 50 mL degassed water and heated to 80 °C under N_2 . 10 ml, 17.5% $\text{NH}_3 \cdot \text{H}_2\text{O}$ was quickly added to the flask while stirring at 800 rpm. The reaction was left for 1 hour at 80 °C before 2 g of Dex-DVS was added. The solution was left to stir overnight before the resulting IOPs-DVS particles were purified by washing with water whilst using a magnet to minimize particle loss. The particles were dried under a flow of N_2 gas.

Synthesis of E_3 or K_3 conjugated magnetic particles (IOPs- E_3 /IOPs- K_3)

Ac- $\text{E}_3\text{GW-PEG}_4\text{-Cys}$ or Ac- $\text{K}_3\text{GW-PEG}_4\text{-Cys}$ was dissolved in PBS (pH 7.4) to a final concentration of 1 mM (as determined by UV-Vis) and then added to 100 mg of IOPs-DVS. The mixture was shaken at 600 rpm at room temperature overnight. The peptide solution was collected under an external magnetic field and the concentration of unreacted peptide was measured by UV-Vis (Figure S18). The difference in peptide concentration before and after the reaction can be used to calculate the amount of peptide conjugated to the magnetic IOPs, (for further details, see the Supporting Information). It was determined that 5.5 nmol peptide E_3 and 5.8 nmol peptide K_3 were conjugated to 1 mg of IOPs. The peptide-functionalized IOPs were purified by washing with H_2O under an external magnetic field. The mass of particles was clearly visible to the naked eye, (Figure S19A) and TEM analysis revealed them to be nanometer sized, (Figure S19B). Particles were stored in 75% EtOH to minimize bacterial growth. Before cell experiments, the particles were washed with PBS and diluted to a final concentration of 2 mg/mL, which equates to 11 μM E_3 in IOPs- E_3 suspension or 11.6 μM K_3 in IOPs- K_3 suspension.

Lipopeptide-decorated cell sorting

Cells were seeded in a six-well plate (1×10^6 cells per well) and incubated overnight. Before trypsinization from the plate, cells were stained with CellTracker™ Green ($5 \mu\text{g/ml}$) or CellTracker™ Red ($10 \mu\text{g/ml}$) for 30 min. 3×10^5 green cells were incubated with 1 mL, $10 \mu\text{M}$ CPK₃ for one hour and washed three times before mixing with the same amount of non-functionalized red cells. 100 μL of IOPs-E₃ was subsequently added to 1 mL cell mixture to facilitate the formation of a coiled-coil between K₃-functionalized cells and the magnetic IOPs. A magnet was used to separate the cells which were connected to the IOPs from the remainder of the cell mixture. The cells in the supernatant were collected and cells connected to the IOPs were then washed three times with PBS before trypsin was added to digest the peptides, (Figure S20 & 21) and dissociate the cells from the IOPs. After detachment, the cells were separated from the IOPs by application of an external magnetic field. Both cell populations were washed twice with PBS before being analyzed. To probe the utility of IOPs functionalized with K₃, the process above was repeated, but cells were decorated with CPE₃ and IOPs were labelled with K₃ in the same manner as described above.

Fluorescence spectroscopy

Fluorescence measurements were performed using a Tecan Infinite M1000 plate reader. All spectra were collected with 200 μL , 2mg/mL IOPs at room temperature in black 96-well plates. For tryptophan fluorescence measurements, excitation was performed at 275nm and emission was recorded from 450 to 310 nm.

In the IOPs fluorescent labeling assay, 200 μL , $10 \mu\text{M}$ fluo-E₃ or fluo-K₃ were added to non-functionalized IOPs or IOPs with a complimentary coiled-coil-forming peptide. After a one minute incubation, the IOPs were thoroughly washed and resuspended in PBS. The IOPs were transferred to a black 96-well plate and a spectrum was recorded. Excitation was performed at 488 nm and the emission spectrum was recorded between 650 and 510 nm.

Confocal microscopy

Cell imaging was performed using a Leica SPE laser scanning confocal microscope. Cells expressing GFP, or stained with CellTracker™ Green or labelled with fluo-K₃/E₃ were excited with a 488 nm laser and the emission signal was detected from 495 to 530 nm. Cells stained with CellTracker™ Red were excited

with a 532 nm laser and emission was detected between 560-600 nm. Cells labelled with Cy5-E₃ were excited with a 635 nm laser and emission was detected from 650-690 nm.

Flow cytometry

All flow cytometry measurements were performed with a Guava® EasyCyte 12HT Benchtop Flow Cytometer and the data was analyzed using FlowJo v10. The cells were suspended in PBS containing 2 mM EDTA at a concentration of approximately 500 cells/μL. 5000 events in duplicate were collected for each measurement. A manual gating strategy can be found in supporting information (Figures S4-S6 & S8-S11). Quadrant gates were used to quantify the fluorescence of Cell Tracker Red (RED-B) *versus* Cell Tracker Green (GRN-B) or GFP expression (GRN-B) *versus* cell size (FSC-A). No compensation was required for the fluorophores used.

Plasmid Constructs

A DNA fragment coding for a signal peptide sequence from the mouse IgK gene and the K₃ peptide fused to a transmembrane domain from PDGFRB, and EGFP was purchased from BaseClear (Leiden, NL) and cloned into an Acc65I and NotI digested pEBMulti-Hyg vector (Wako Pure Chemical Ind, Osaka, Japan) as described previously.³⁷ The DNA sequence is shown in the Supporting Information (Figure S22).

Cell transfection and antibiotics selection

HeLa and NIH3T3 cells were seeded in a 12-well plate and grown to 80% confluency. 1 μg of plasmid (0.2 μg/μL) and 8 μg of PEI were used per well. The cells were incubated with the DNA/PEI complex for 5 hours at 37 °C and then washed with DMEM three times.

CHO cells were transfected using Lipofectamine 3000. Cells were seeded in a 24-well plate and allowed to grow to 80% confluency. 0.5 μg of plasmid DNA (0.2 μg/ μL) and 1.5 μL of Lipofectamine 3000 were used per well. Cells were incubated at 37 °C for 5 hours before washing the cells with DMEM three times.

After transfection, all cells were grown for three days. Hygromycin B was used to enrich successfully transfected cells. After two weeks of antibiotic selection, the percentage of GFP-positive cells was found to stop increasing, presumably because

the cells acquired resistance to the antibiotic. At this point, the GFP-expressing cells comprised approximately 10% of the total cell population.

MACS with GFP-K₃ expressed cells

Before MACS, GFP-K₃ expressing cells were subcultured for at least two generations. The cells were subsequently detached from the cell culture plate using EDTA (2 mM in PBS) and dispersed by thorough pipetting. Cell sorting was performed by utilizing 1 mL cell suspension containing 1×10^6 GFP-K₃ expressing cells. 100 μ L of IOPs-E₃ suspension was subsequently added to enable coiled-coil formation between the K₃ peptide on the cell membrane and the E₃ peptide attached to the IOPs. A magnet was then used to separate the cells which were connected to the IOPs from the other cells in the mixture. Cells connected to the IOPs were washed with PBS three times and trypsin was then added to dissociate the cells from the IOPs. After detachment, the cells and IOPs were separated by application of an external magnetic field.

Transmission Electron Microscopy

A 10 μ L droplet of the IOPs was placed on a Forvar/Carbon grid (200 mesh) and left for ten minutes. The excess solution was blotted off and the grid was left to air dry. Images were obtained using a JEM1400 plus (JEOL) microscope, operating at 80 kV. The microscope was fitted with a CCD camera.

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APPENDIX 2

Cell Labelling Experiments

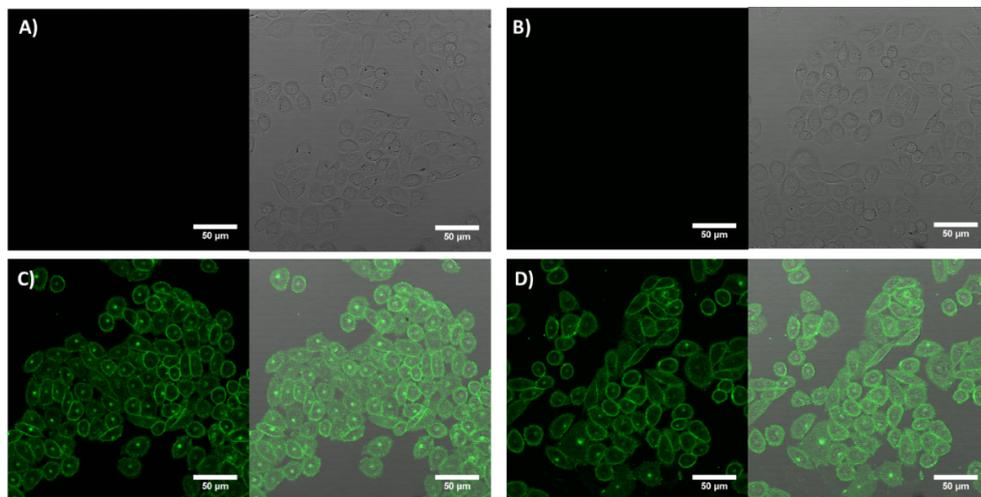


Figure S1. Cell labeling assay with HeLa cells. A) Non-labeled cells incubated with fluo-K₃, B) non-labeled cells incubated with fluo-E₃, C) CPE₃ decorated cells incubated with fluo-K₃, D) CPK₃ decorated cells incubated with fluo-E₃. Green: fluorescein. Scale bar: 50 μm.

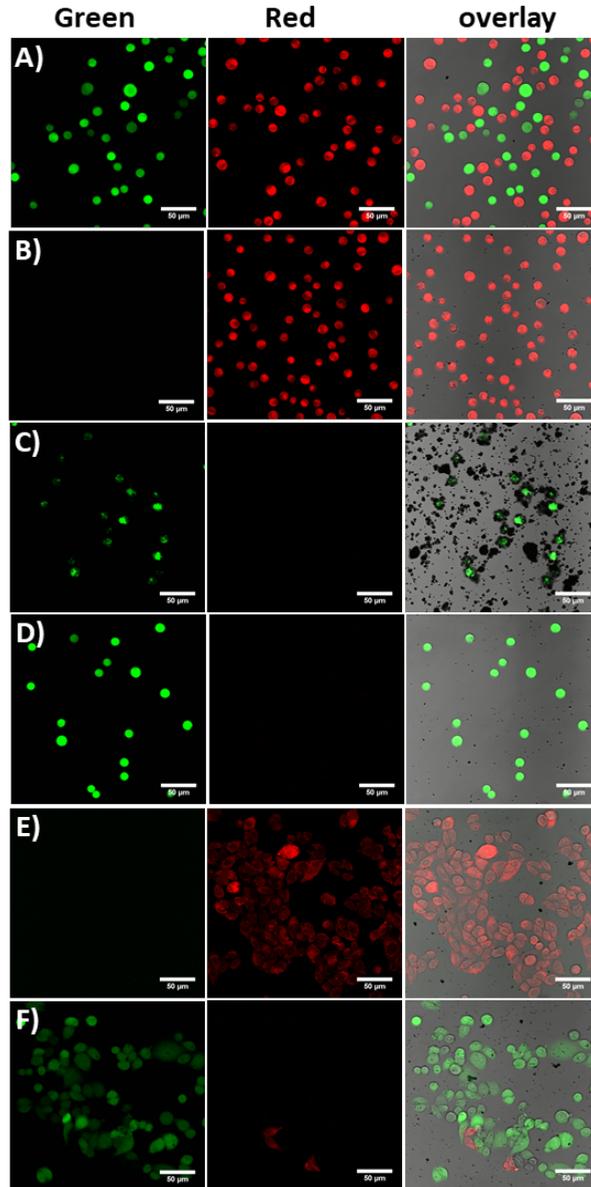
MACS with CHO and NIH3T3 cells—CPK₃ decorated cells

Figure S2. CHO cell sorting using IOPs-E₃ A) Green cells were decorated with CPK₃ and mixed with the same number of red cells. (B) Cells in the supernatant after MACS. (C) Cells attached to IOPs after MACS. (D) Cells detached from IOPs by trypsinization. (E) Cells in the supernatant 24 hours after MACS and (F) cells detached from the IOPs 24 hours after MACS. Cells were stained with CellTracker Green or CellTracker Red. Scale bar 50µm.

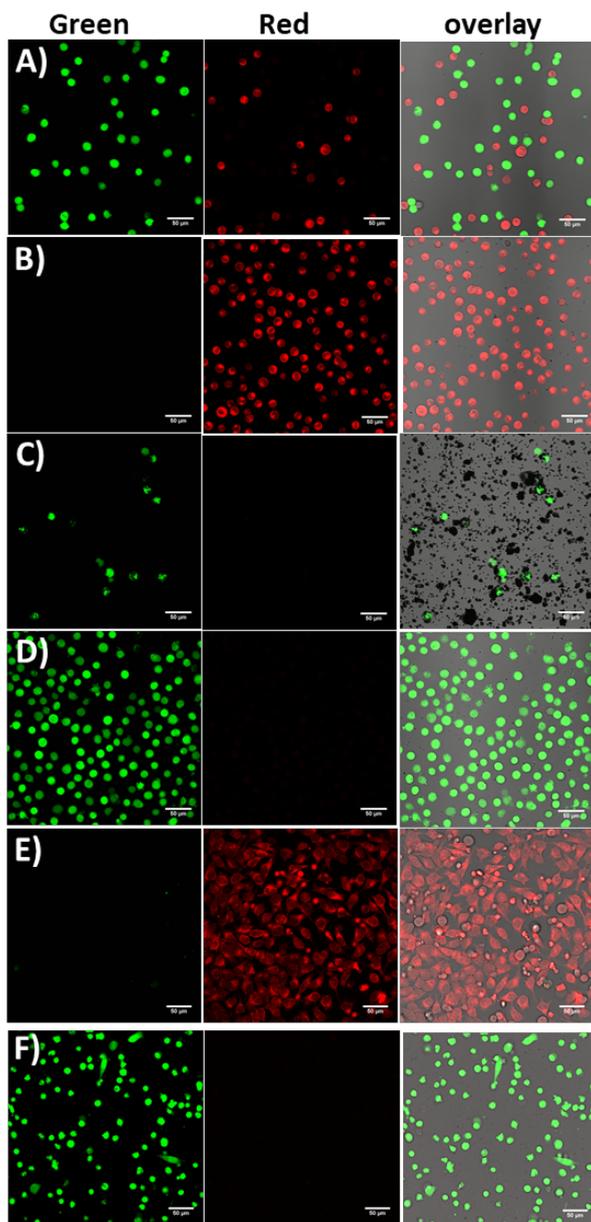


Figure S3. NIH3T3 cell sorting using IOPs-E₃ A) Green cells were decorated with CPK₃ and mixed with the same number of red cells. (B) Cells in the supernatant after MACS. (C) Cells attached to IOPs after MACS. (D) Cells detached from IOPs by trypsinization. (E) Cells in the supernatant 24 hours after MACS and (F) cells detached from the IOPs 24 hours after MACS. Cells were stained with CellTracker Green or CellTracker Red. Scale bar 50µm.

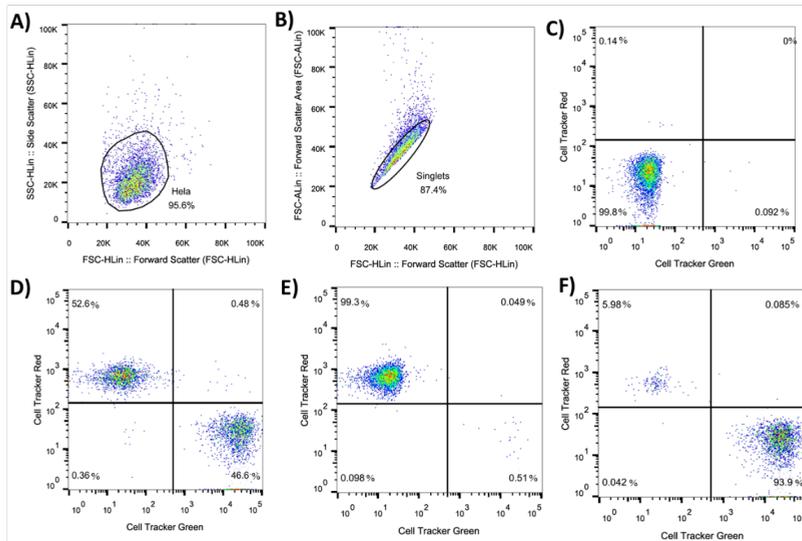
FACS analysis of cell population after MACS with CPK₃ decorated cells

Figure S4. HeLa cell sorting using IOPs-E3. A) HeLa cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) HeLa cells without staining, D) HeLa cells mixture: green-CPK₃ and red cells, E) cells in supernatant after MACS, F) cells detached from IOPs after MACS. Cell tracker Green: GRN-B, cell tracker Red: RED-B.

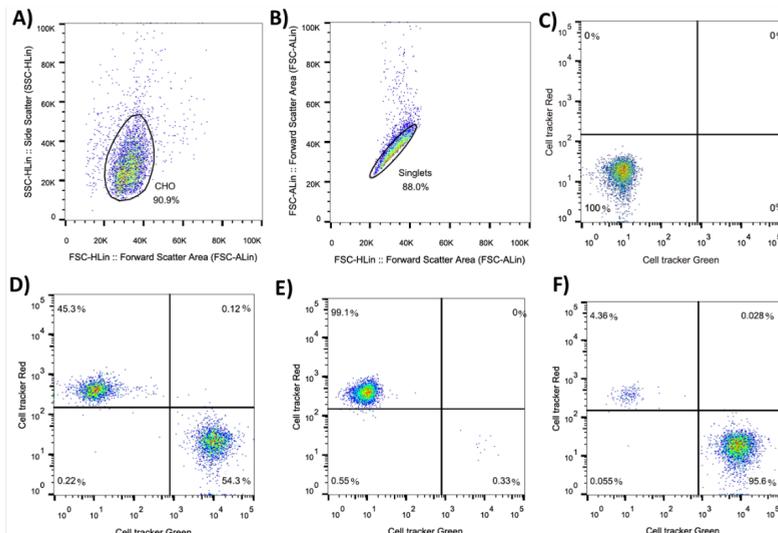


Figure S5. CHO cell sorting using IOPs-E3. A) CHO cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) CHO cell without staining, D) CHO cells mixture: green-CPK₃ and red cells, E) cells in supernatant after MACS, F) cells detached from IOPs after MACS. Cell tracker Green: GRN-B, cell tracker Red: RED-B.

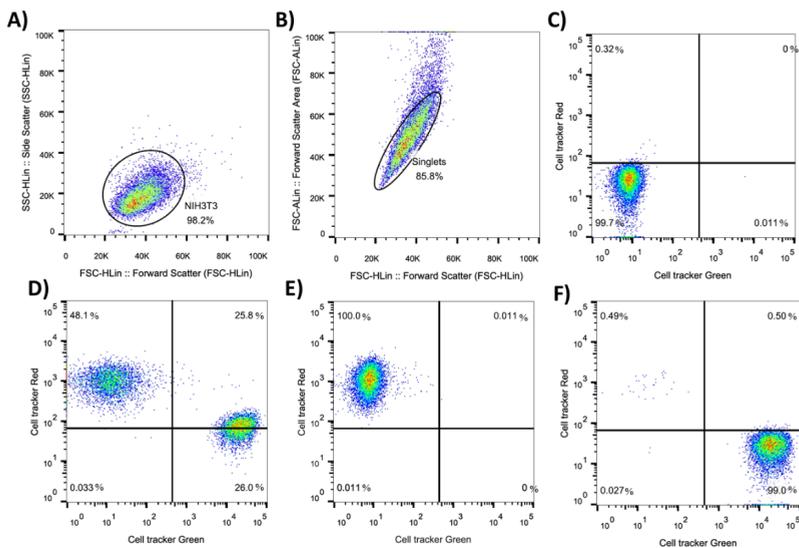


Figure S6. NIH3T3 cell sorting using IOPs-E3. A) NIH3T3 cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) NIH3T3 cell without staining, D) NIH3T3 cells mixture: green-CPK₃ and red cells, E) cells in supernatant after MACS, F) cells detached with IOPs after MACS. Cell tracker Green: GRN-B, cell tracker Red: RED-B.

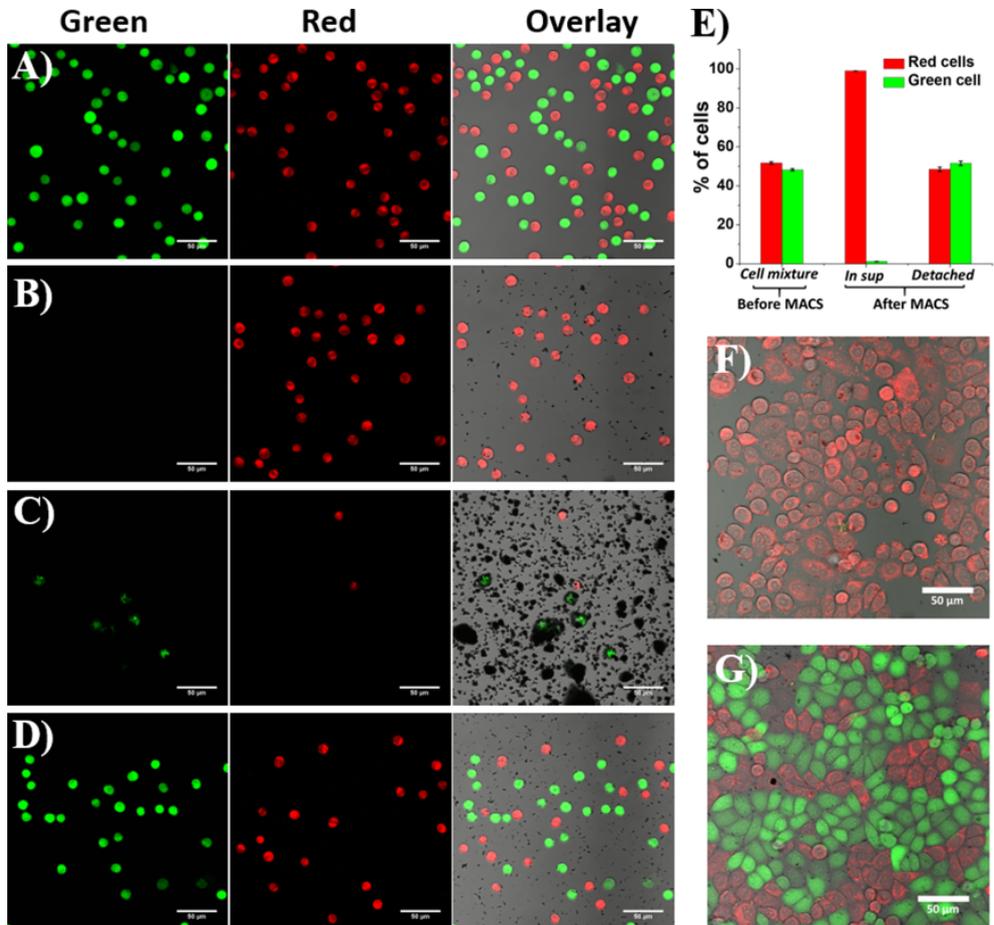
MACS with peptide K_3 functionalized IOPs

Figure S7. Coiled-coil-assisted MACS of HeLa cells. A) CPE₃-modified HeLa cells stained with CellTracker™ green were mixed with HeLa cells stained with CellTracker™ red; B) cells in the supernatant after MACS; C) IOP-attached cells after MACS; D) detached cells after MACS. Scale bar: 50 μ m. E) The bar chart illustrates cell quantification, *via* FACS, before and after MACS. Cells from the supernatant (F) and those detached from the IOPs (G) show signs of recovery and growth after 24 hours. Scale bar: 50 μ m.

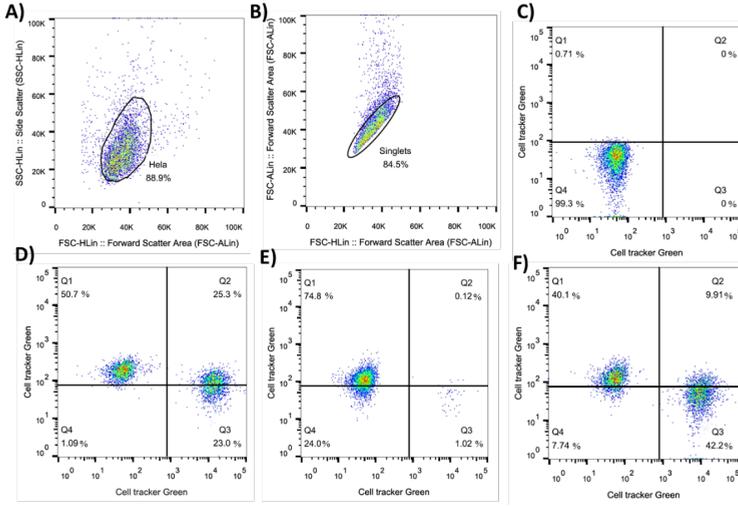


Figure S8. HeLa cell sorting using IOPs- K_3 . A) HeLa cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) HeLa cell without staining, D) HeLa cells mixture: green-CPE₃ and red cells, E) cells in supernatant after MACS, F) cells detached with IOPs after MACS. Cell tracker Green: GRN-B, cell tracker Red: RED-B.

FACS analysis of cell population after MACS with K_3 membrane expressed cells

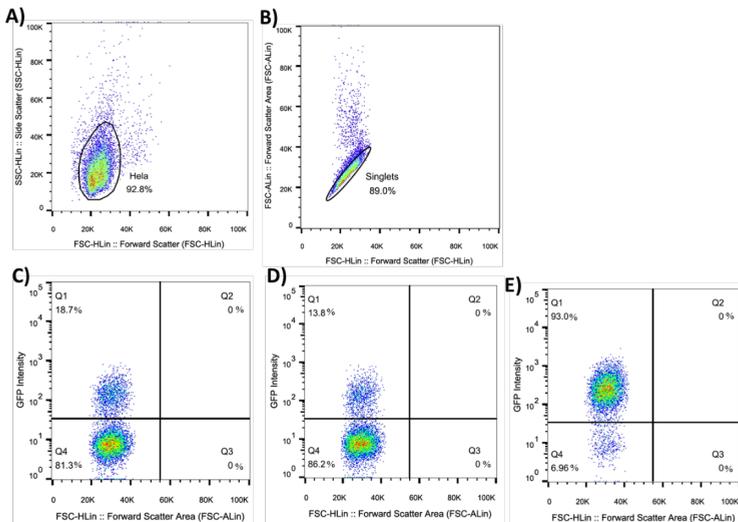


Figure S9. HeLa-GFP- K_3 cells enrichment by MACS. A) HeLa-GFP- K_3 cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) Cells after transfection and two weeks antibiotics selection. D) Cells in supernatant after MACS, E) cells detached from IOPs after MACS. GFP: GRN-B.

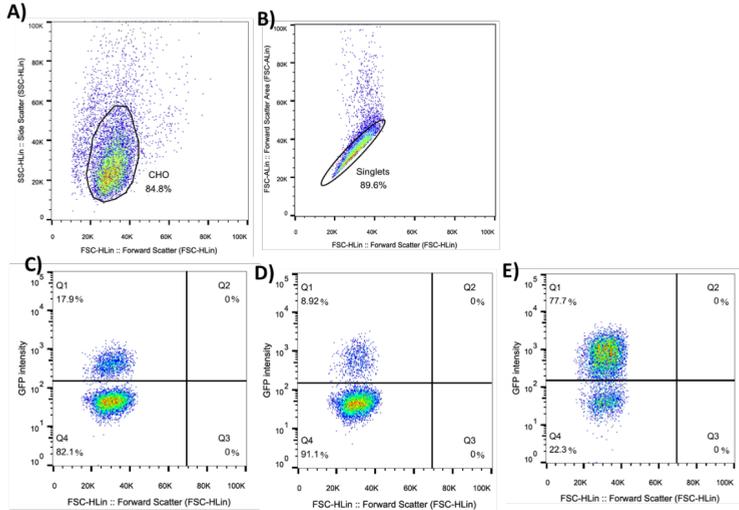


Figure S10. CHO-GFP-K₃ cells enrichment by MACS. A) CHO-GFP-K₃ cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) Cells after transfection and two weeks antibiotics selection. D) Cells in supernatant after MACS, E) cells detached from IOPs after MACS. GFP: GRN-B.

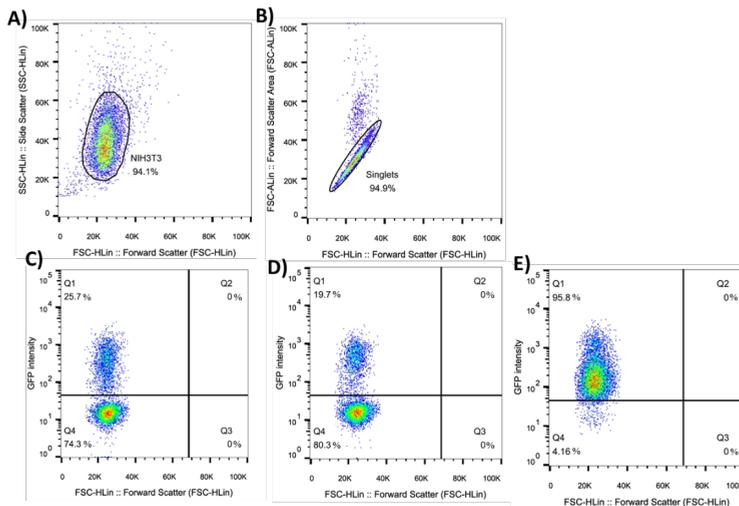
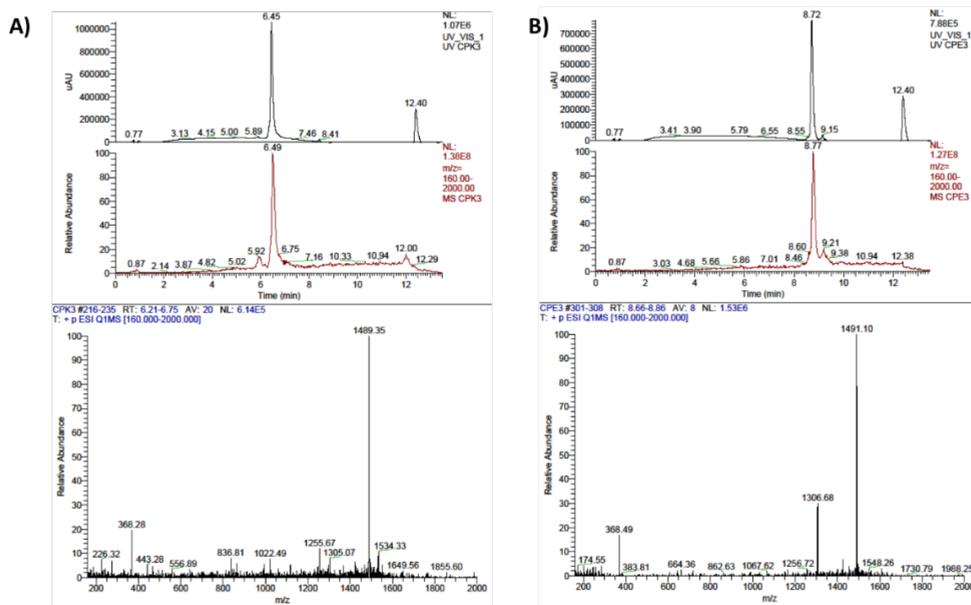


Figure S11. NIH3T3-GFP-K₃ cells enrichment by MACS. A) NIH3T3-GFP-K₃ cell population gated by FSC-H vs. SSC-H, B) cell singlets gated by FSC-A vs. FSC-H, C) Cells after transfection and two weeks antibiotics selection. D) Cells in supernatant after MACS, E) cells detached from IOPs after MACS. GFP: GRN-B.

LC-MS data for the synthesized peptides.

Table S1. Calculated mass and found mass via LC-MS of all peptides

peptide	Mass (calcd.)	Mass (found)
CPK ₃	2978.0	2977.7
CPE ₃	2980.8	2981.2
Ac-K ₃ GW-PEG ₄ -Cys	2898.7	2899.2
Ac-E ₃ GW-PEG ₄ -Cys	2901.5	2901.2
Fluo-K ₃	2969.6	2969.6
Fluo-E ₃	2972.5	2972.0
Cy5-E ₃	3305.8	3306.8

Figure S12. LC-MS of: A) CPK₃ and B) CPE₃.

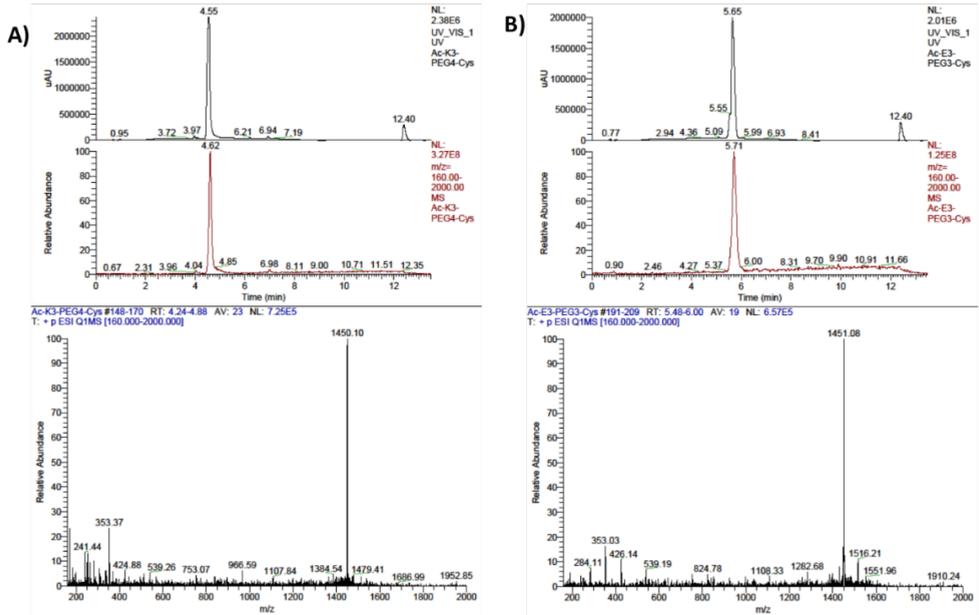


Figure S13. LC-MS of: A) Ac-K₃GW-PEG4-Cys and B) Ac-E₃GW-PEG4-Cys.

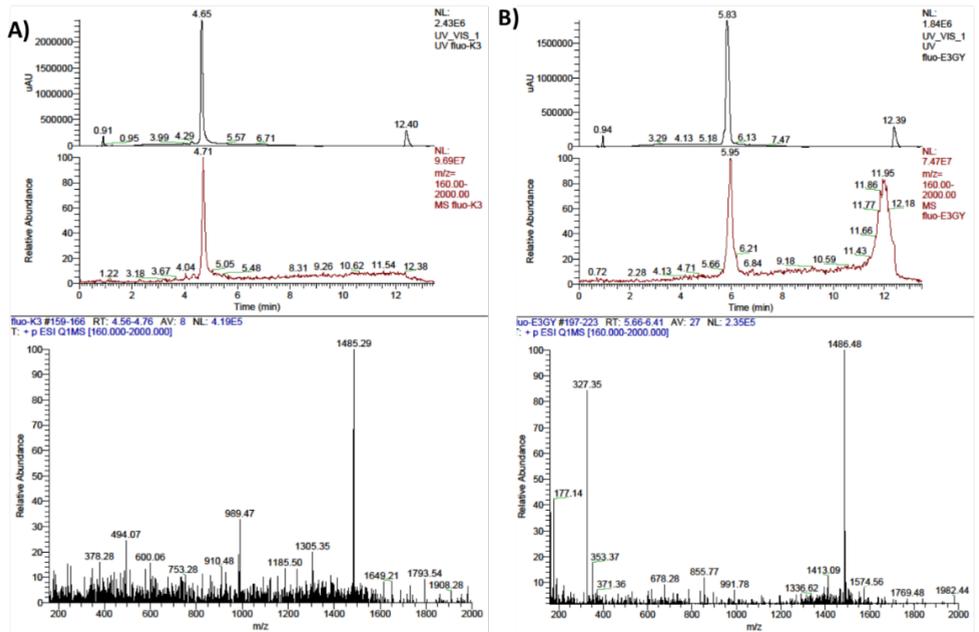


Figure S14. LC-MS of: A) fluo-K₃ and B) fluo-E₃.

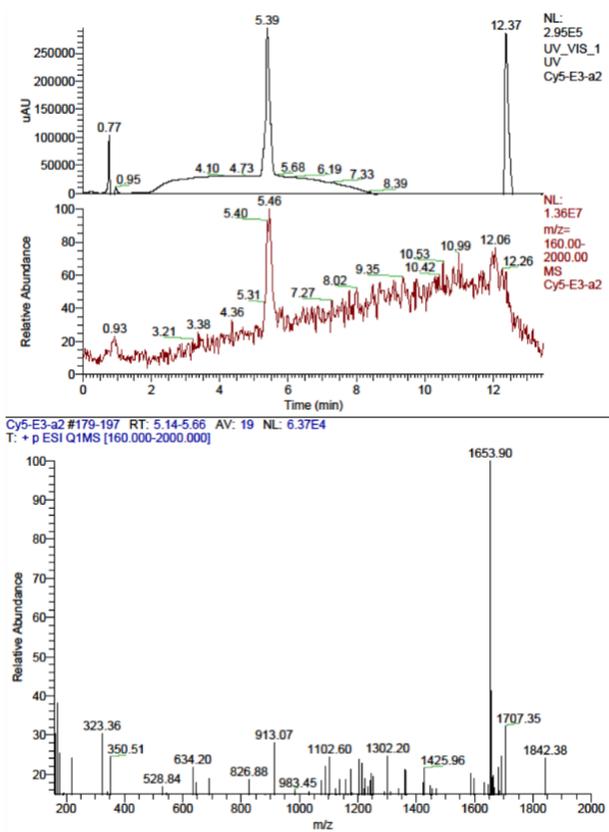


Figure S15. LC-MS of Cy5-E₃.

Synthetic, coating and functionalize IOPs

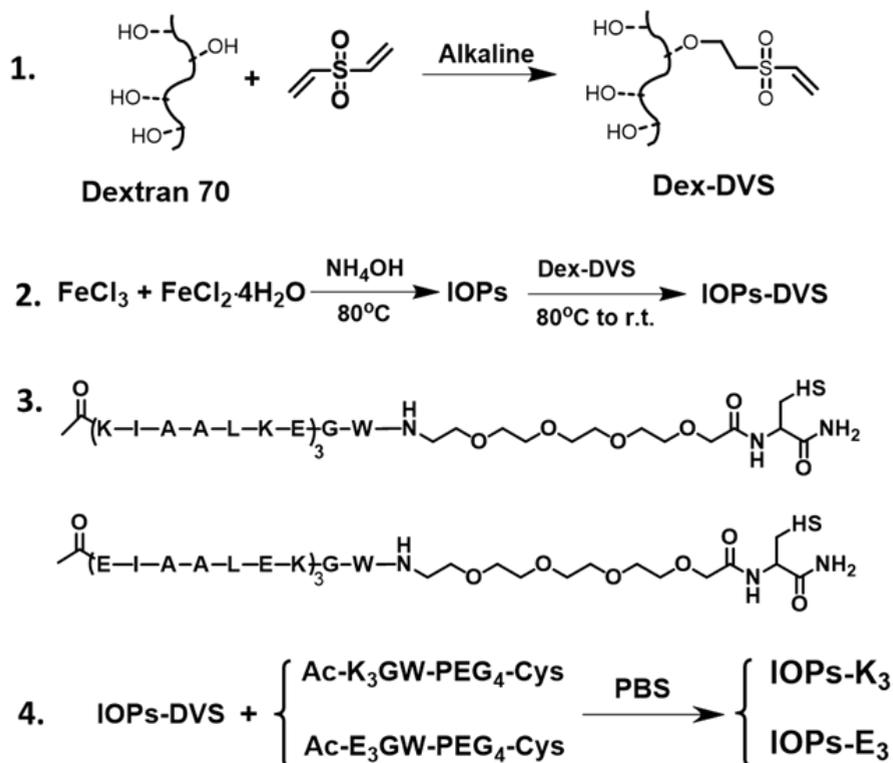


Figure S16. Synthetic route to coiled-coil functionalized magnetic particles: 1. Synthesis of Dex-DVS, the NMR data of Dex-DVS can be seen in Figure S16. 2. Synthesis of micron-sized IOPs and surface coating. 3. Chemical structure of Ac-K₃-PEG₄GW-Cys and Ac-E₃GW-PEG₄-Cys, 4. IOPs-DVS functionalization, coiled coil peptide conjugated to the IOPs-DVS by Michael addition between vinyl sulfone group on dextran-DVS and sulfhydryl group on cysteine.

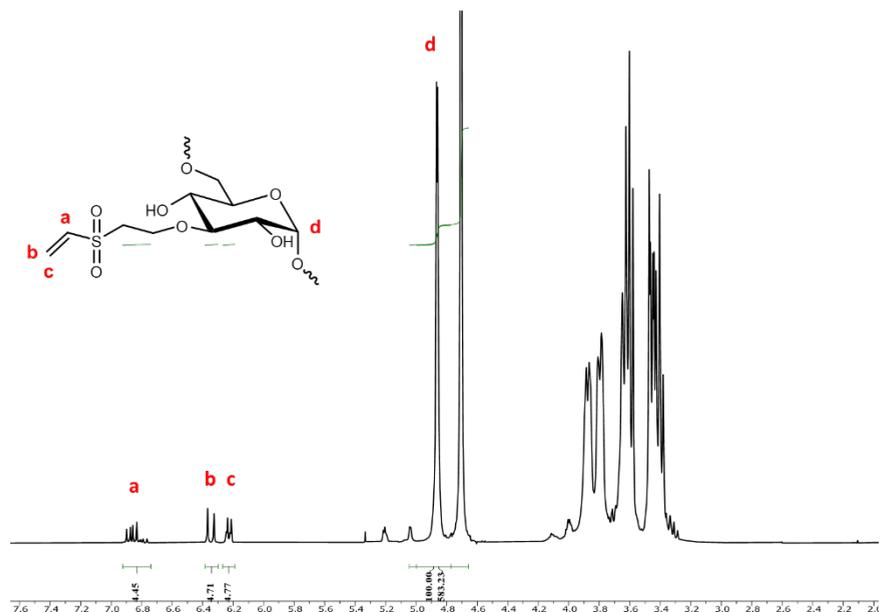


Figure S17. ^1H NMR of Dex-DVS used to coat IOPs. ^1H NMR (400MHz, D_2O): δ 3.2-4.2 (m, dextran glucosidic protons) 4.9 (s, dextran anomeric proton), 6.8-6.9 (m, $-\text{SO}_2\text{CH}=\text{CH}_2$), 6.2 (d, $-\text{SO}_2\text{CH}=\text{CH}_2$), 6.4 (d, $-\text{SO}_2\text{CH}=\text{CH}_2$). The integral of peak 'd' can be used to define the total number of d-glucopyranose repeating units in dextran. The integrals of peaks 'a' 'b' or 'c' can be used to define the number of vinyl sulfone modified d-glucopyranose repeating units. Here, the integral of peak 'd' has been defined as 100, then the average integral of peaks 'a' 'b' and 'c' can be calculated and this corresponds to the degree of substitution (DS). The DS of the Dex-DVS used in this study is 4.6.

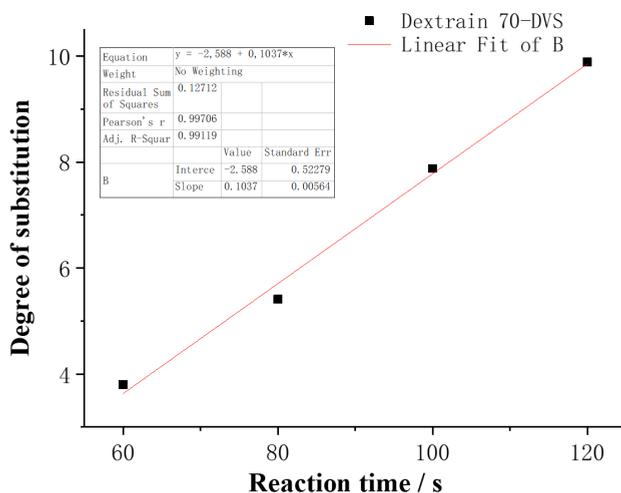


Figure S18. The linear fit which used determine the DS of dextran-DVS with reaction time.

Determination of peptide concentration attached to the IOPs.

The density of peptides conjugated to the IOPs can be calculated using equation 1.

$$\rho = \Delta A \cdot V_1 \cdot 10^6 / (\varepsilon \cdot l \cdot m_1) \quad (1)$$

ρ is the density of peptide on the IOPs (nmol/mg), ΔA is the difference in UV-Vis absorption at 280nm before and after IOPs functionalization, V_1 is the volume of peptide solution reacted with IOPs (mL), ε is the molar absorption coefficient of tryptophan at 280nm ($M^{-1} \text{ cm}^{-1}$), l is path length of cuvette (cm), m_1 is the mass of IOPs reacted with peptide (mg).

Since the concentrations of peptide used to functionalize IOPs are too high to directly measure, both peptide Ac-E₃GW-PEG₄-Cys and Ac-E₃GW-PEG₄-Cys have been diluted four times before the UV-Vis measurement was performed. The calculated ρ for IOPs-E₃ is 5.5 nmol/mg and for IOPs-K₃ is 5.8 nmol/mg.

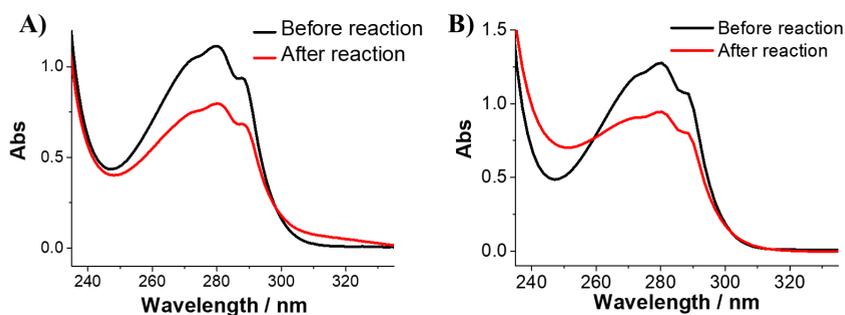


Figure S19. UV-Vis spectra of Ac-E₃GW-PEG₄-Cys A) and Ac-K₃GW-PEG₄-Cys B) before and after reacting with IOPs-DVS.

9. Images of IOPs-E₃

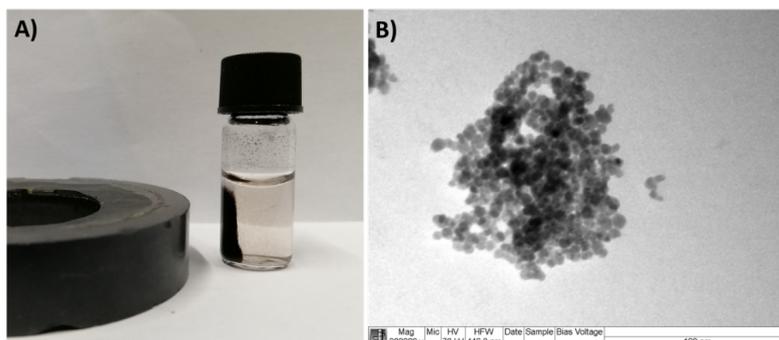


Figure S20. A) Image of IOPs-E₃ with an external magnetic field applied. B) TEM image of IOPs-E₃.

10. Trypsin degradation of peptide Ac-K₃GW-PEG₄-Cys and Ac-E₃GW-PEG₄-Cys

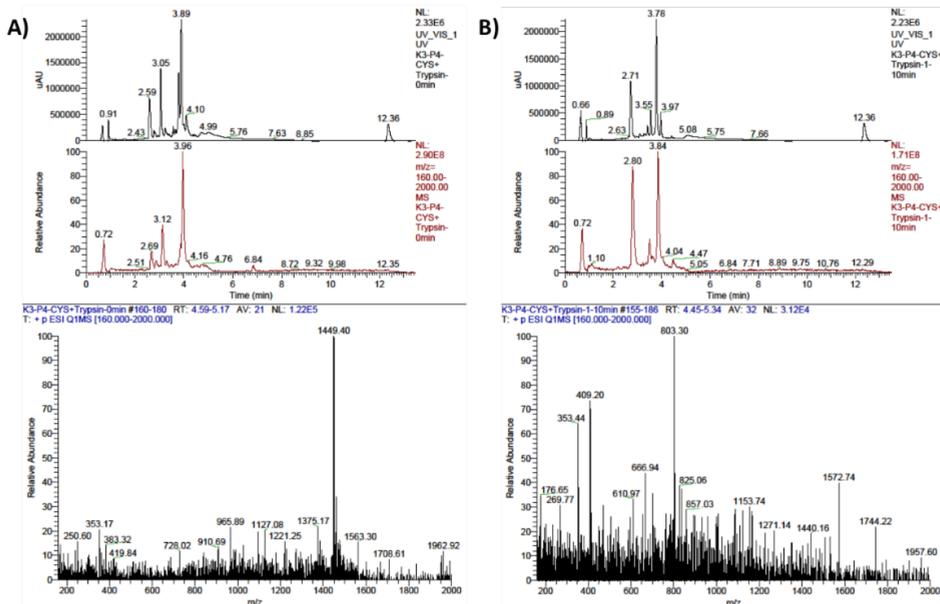


Figure S21. LC-MS of Ac-K₃GW-PEG₄-Cys digested by trypsin. Ac-K₃GW-PEG₄-Cys was incubated with trypsin for: A) 0 min and B) 10 min at 37 °C.

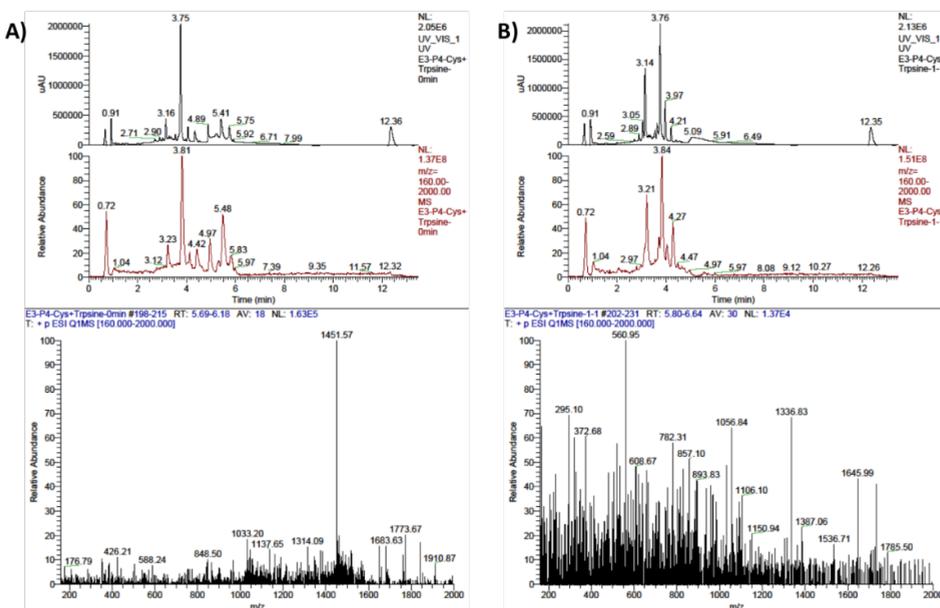


Figure S22. LC-MS of Ac-E₃GW-PEG₄-Cys digested by trypsin. Ac-E₃GW-PEG₄-Cys incubated with trypsin for: A) 0 min and B) 10 min at 37 °C.

11. DNA sequence of the K3-TMD-GFP gene

ggtaccGCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGC
 TCTGGGTTCAGGTTCCACTGGTGACGGTGGgtcgactAAAATAGCCGC
 ACTGAAGGAAAAAATCGCTGCGCTGAAGGAGAAGATTGCTGCACTC
 AAGGAGgtgaccAACGCTGTGGGCCAGGACACGCAGGAGGTCATCGTG
 GTGCCACACTCCTTACCGGTAAAGGTGGTGGTGATCTCAGCCATCCT
 GGCCCTGGTGGTGCTCACCATCATCTCCCTTATCATCCTCATCATGCT
 TTGGCAGAAGAAGCCACGTGGTGGAtgtacaGTGAGCAAGGGCGAAGA
 GCTGTTACCCGGGGTGGTGCCATCCTGGTTCGAGCTGGACGGCGACG
 TAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGC
 CACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGC
 TGCCCGTGCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCGTG
 CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTT
 CAAGTCCGCCATGCCCGAAGGCTACGTCCAAGAGCGCACCATCTTCT
 TCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGA
 GGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTC
 AAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACA
 ACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCAT
 CAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTG
 CAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCC
 CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTG
 AGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGT
 TCGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTATAAG
 TAActcgagattaatgcggccgc

Figure S23. The DNA sequence of the K₃-TMD-GFP gene comprises a signal sequence from the mouse IgK gene (yellow), the K₃ peptide (red), a transmembrane domain from PDGFRB (blue), and EGFP (green).

