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Superparamagnetic core–shell nanoparticles as solid supports for peptide synthesis†

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Functional superparamagnetic core–shell nanoparticles are synthesized by a microwave assisted route and can be used as colloidal supports for peptide synthesis in "quasi solution".

In 1963 Merrifield *et al.* introduced the method of solid-phase supported synthesis and thus revolutionized peptide synthesis.[1] Nowadays, solid-phase supported peptide synthesis (SPPS) is a fully automated process with commercially available reagents and α-amino acid derivatives.[2] SPPS provided models to deepen insight into protein function and paved the way to peptide drugs.[3] Even material scientists used this platform to synthesize sequence-defined polymers for bioinspired soft-matter science.[4]

Practically all reagents and components used in SPPS were optimized. For instance, the solid supports underwent adaptation from the first modified ion-exchange resins to modern hybrid microparticles *e.g.* TentaGel® resins.[3b,5] However, conceptually not much has been changed compared to the first proof-of-principle study. Still lightly cross linked poly(styrene) resins of 70–120 μm dominate the used supports. Certainly, established microgels have high capacity and allow ease of purification by filtration. However, the accessibility of functionalities requires particle swelling and the diffusion of reagents within the microgel is limited. Solid 100 μm glass beads exhibit improved accessibility to surface functionalities, but suffer from low capacity. Nanoparticles overcome this problem as *e.g.* ~70 nm particles span a $\sim 1.1 \times 10^5$-times higher surface area. Routes to nanoparticles of various materials are established, offering large scale access.[6] However, filtration procedures are not practical if nanoparticles were applied as supports. Within recent decades, magnetic sedimentation of colloids by external magnetic fields has become a useful tool to selectively pull-down compounds out of complex mixtures.[6a,7] Only few reports on the peptide synthesis with magnetic supports appeared, but the fundamental concept of SPPS supports was not evolved.[8]

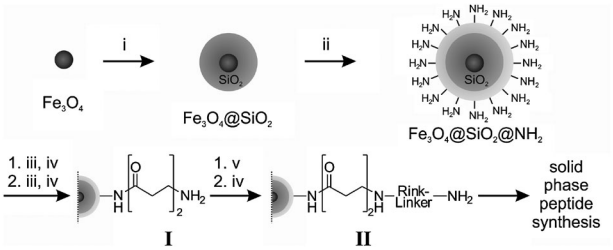

Fig. 1 Synthesis of core–shell nanoparticles with amino functionalities at the surface. (i) TEOS, NH_3/H_2O/ethanol, 60 °C, 3 min, microwave; (ii) APTMS, NH_3/H_2O/ethanol, 60 °C, 3 min, microwave; (iii) Fmoc-β-Ala OH, PyBOP/DIPEA/NMP, 3 h; (iv) piperidine/NMP, 5 min; (v) Fmoc-Rink-Linker OH, PyBOP/DIPEA/NMP, 3 h.

Here, we report on surface amino functionalized, superparamagnetic nanoparticles with a protective silica shell to be applicable as colloidal supports for peptide synthesis in "quasi solution" (Fig. 1). The absence of diffusion limited reactions in microgel environments and permanent surfaces enable direct access to loci of synthesis. Convenient magnetic sedimentation proved to ensure ease of purification after each reaction step.

Sequential assembly of peptides requires repetitive coupling and deprotection cycles to be performed on the support and purification of the intermediate products in-between each cycle is necessary. As magnetic sedimentation of magnetite nanoparticles works in various solvents, superparamagnetic magnetite particles were synthesized. A microwave facilitated route enabled rapid synthesis of monodisperse magnetite nanoparticles from iron(III)acetylacetonate.[9] Well-defined, monodomain magnetite nanoparticles could be isolated from the resulting homogeneous suspension by magnetic sedimentation. Transmission electron microscopy (TEM) images showed well-dispersed, uniform monodomain nanoparticles with defined crystal structure and widths of 6 ± 1 nm (Fig. 2a, and Fig. S1, ESI†). Atomic force microscopy (AFM) confirmed an average particle diameter of 9 ± 3 nm (Fig. S2, ESI†). Powder X-ray diffraction (XRD) patterns suggested the presence of phase-pure magnetite with crystallite sizes of ~9 nm as estimated from the line widths of the reflections (Fig. S3, ESI†).

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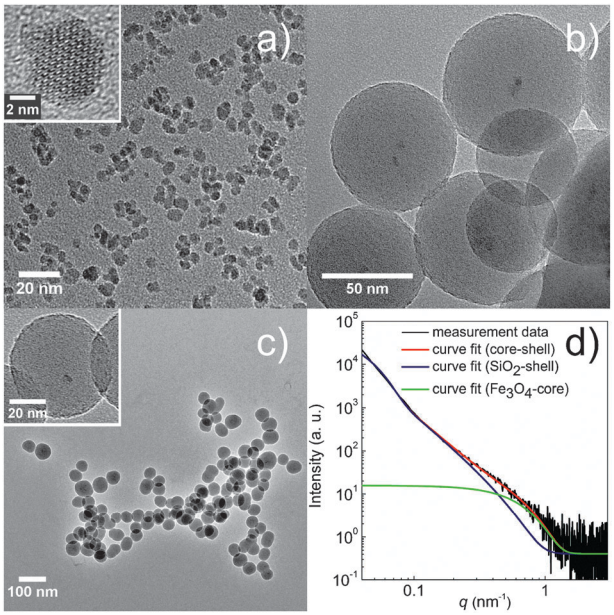

Fig. 2 Analysis of seed and core–shell particles: TEM of (a) Fe_3O_4 (inset HRTEM), (b) Fe_3O_4@SiO_2 and (c) Fe_3O_4@SiO_2@NH_2 particles. SAXS of Fe_3O_4@SiO_2@NH-(β-Ala)$_2$-Rink particles (d).

The sensitivity of magnetite particles against both acidic and basic conditions, which will be utilized during peptide synthesis, makes protection essential. A sufficiently thick silica shell was constructed around the magnetite nanoparticles (Fig. 1), using a rapid microwave assisted Stöber method with tetraethoxysilane (TEOS) as a precursor.[10] The magnetite seed particles were readily dispersed in ethanol–water with TEOS and ammonia. After 3 min microwave irradiation at 60 °C, monodisperse core–shell Fe_3O_4@SiO_2 particles were isolated by magnetic sedimentation. Zeta-potential measurements indicated qualitatively the successful coating by a change from $\zeta = +44.3$ mV of the magnetite seed particles to $\zeta = -38.1$ mV representative of silica. The chemical identity of the Fe_3O_4@SiO_2 particles was confirmed by Fourier transform infrared (FTIR) spectroscopy, revealing an absorption at $\nu = 1063$ cm^{-1} that is typical for SiO_2 (Fig. S6 and S12, ESI†). TEM proved the formation of defined core–shell particles, with a size of 67 ± 9 nm (Fig. 2 and Fig. S5, ESI†). Energy-dispersive X-ray spectroscopy (EDX) indicated an increased percentage of Si and O after coating (Table S1, ESI†).

To verify the desired shielding effect and indirectly to confirm the integrity of the silica shell around the magnetite core, the Fe_3O_4@SiO_2 particles were exposed to harsh conditions used during SPPS. A treatment with piperidine in *N*-methyl-2-pyrrolidone (20 vol%) for several days could not harm the core–shell particles, while uncoated magnetite dissolves within minutes. The stability under acidic conditions was proved by treating the particles with trifluoroacetic acid in dichloromethane (50 vol%). UV/vis-spectroscopy was used to follow the development of soluble iron compounds that could not be magnetically sedimented (Fig. S10, ESI†). While the core–shell particles showed excellent stability for at least 3 h, the pure magnetite particles instantly started to bleed-out soluble iron compounds.

The required functionalization of the Fe_3O_4@SiO_2 particle surface with amino groups was realized in a secondary Stöber process with (3-aminopropyl)trimethoxysilane (APTMS) as a precursor. In contrast to many time-consuming literature procedures, microwave assisted APTMS hydrolysis and condensation lead in ~3 min at 60 °C to the functional coating on the surface of Fe_3O_4@SiO_2 particles. ζ-Potential analysis gave a qualitative verification of the successful surface functionalization by showing a change from $\zeta = -38.08$ mV for Fe_3O_4@SiO_2 to $\zeta = +41.14$ mV for Fe_3O_4@SiO_2@NH_2 nanoparticles. TEM micrographs showed the preservation of the core–shell structure and the absence of obvious particle agglomeration during coating (Fig. S7, ESI†). Analysis of the TEM images revealed rather defined particles with a slightly increased size of 69 ± 8 nm (Fig. 2c). Small-angle X-ray scattering (SAXS) confirmed these dimensions and the core–shell structure by excellent curve fits based on the dimensions derived from HRTEM (Fig. 2d, Fig. S13, ESI†). EDX proved functionalization by indicating an increase of carbon and nitrogen content (Table S1, ESI†). To accurately determine the concentration of available amino groups on the Fe_3O_4@SiO_2@NH_2 particle surface, a quantitative 9*H*-fluoren-9-yl-methoxycarbonyl (Fmoc)-test was performed.[11] Coupling Fmoc-protected glycine to the particles and subsequent cleavage of the protective groups enabled a read-out of the liberated dibenzofulvene-piperidine by UV-spectroscopy. A concentration of 0.11 mmol amino groups per gram of particles could be calculated, which is in the range of common Tentagel® peptide synthesis resins (0.14–0.27 mmol g^{-1}). Probably the loading could be easily increased by successive coupling of multiple Fmoc Lys(Fmoc) OH derivatives. N_2-sorption revealed an active surface area of 30 m^2 g^{-1} for the functionalized particles. Assuming compact spheres with a density of bulk silica allows for the calculation of a particle diameter of 76 nm, which agrees well with the observed $d_{TEM} = 70$ nm. An average of two amino groups per 1 nm^2 on the particle surface could be calculated, assuming 70 nm particles. The obtained loading appears to be a suitable compromise between optimal peptide synthesis and high support capacity. A higher loading will probably increase the risk of peptide–peptide interactions, which might negatively interfere with the peptide synthesis. To reduce unfavourable contacts between peptides and the silica surface that also might prevent effective peptide synthesis a spacer in form of two β-alanine units was attached to the amino functionalities of the Fe_3O_4@SiO_2@NH_2 particles by coupling Fmoc-β-alanines (Fig. 1). Subsequent attachment of a Rink-amide linker moiety introduced the semi-permanent 4-(2′,4′-dimethoxyphenylhydroxymethyl-phenoxy)-linker (Fig. 1 and Fig. S11, ESI†) that can be used to liberate the final peptide from the support. The coupling of the β-alanines and the Rink-linker took already the advantage of magnetic sedimentation to purify the intermediates after each reaction step. FTIR spectroscopy showed the occurrence of characteristic amide bands at $\nu = 1672$ cm^{-1} and 1548 cm^{-1} (Fig. S12, ESI†). Wide angle X-ray scattering (WAXS) data correspond to the calculated magnetite pattern and indicated that the multistep process was not changing the crystal structure of the magnetic core particles (Fig. S8, ESI†). To study the sedimentation behaviour of the colloidal supports, gravitational

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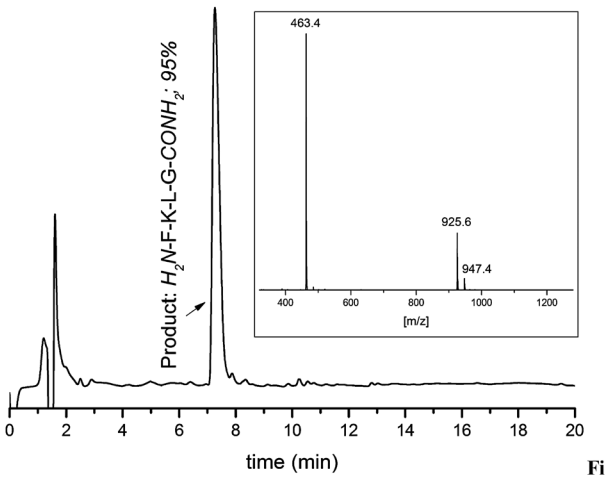

Fig. 3 HPLC-trace and ESI-MS spectrum of synthesized peptide H_2N–Phe-Lys-Leu-Gly–$CONH_2$ (gradient: 3–50% MeCN, RP-C18-column, λ = 210 nm). Mass of peptide found with 95% purity.

and magnetic sedimentation was investigated by turbidity measurements (Fig. S9, ESI†). While the supports sediment in the absence of external magnetic fields within ~4 hours, a strong magnetic field induced rapid sedimentation within ~2 minutes. Redispersion of the sedimented particles occurs upon gentle shaking.

Ultimate proof for the applicability of colloidal supports, however, requires the synthesis of peptides on Fe_3O_4@SiO_2@NH-(β-Ala)$_2$-Rink supports. Two peptides were synthesized using sequential assembly of Fmoc-α-amino acids, implementing colloidal supports to standard SPPS procedures. A magnetic sedimentation step was employed to isolate the intermediate products after each reaction step. According to standard SPPS, coupling reactions were facilitated by benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate with *N*,*N*-diisopropyl-ethylamine in NMP. The deprotection of the Fmoc protected α-amino groups was achieved by piperidine in NMP. As a first model a tetrapeptide with a Phe-Lys-Leu-Gly sequence was synthesized (Fig. 3). Liberation from the support after peptide synthesis was performed with TFA–DCM (50 vol%). Magnetic sedimentation of the empty supports resulted rapidly in a colourless cleavage solution. The peptide could be isolated by ether precipitation with ~70% yields. HPLC-ESI-MS analysis of the crude peptide exhibits remarkable product purity of *ca.* 95% (Fig. 3; Fig. S14 and S15, ESI†).

Moreover, a longer undecapeptide with biological relevance was synthesized to demonstrate the feasibility of the strategy. The sequence Gln-Thr-Thr-Thr-Trp-Gln-Asp-Pro-Arg-Lys-Gly as part of the hYAP WW peptide domain[12] could be obtained. MALDI-TOF-MS proved the identity of the crude peptide and confirmed that longer sequences can be synthesized on colloidal supports, as well (Fig. S16, ESI†). The high purity of both peptides was in the upper range of peptides that can be obtained on common SPPS resins. Only minor amounts of side products could be observed, which are assignable to either deletion sequences or stop sequences, both of which are common side reactions to standard SPPS (Fig. S14–S16, ESI†).

In summary, functional core–shell nanoparticles were synthesized by a microwave assisted route, enabling rapid and reproducible synthesis of well-defined Fe_3O_4@SiO_2@NH_2 nanoparticles. They could be decorated on the surfaces with Rink-amide linkers to make the Fe_3O_4@SiO_2@NH-(βAla)$_2$-Rink core–shell particles applicable as colloidal supports for peptide synthesis. A tetrapeptide model and a potentially bioactive undecapeptide were accessed, both exhibiting excellent purities as isolated crude products. As the magnetic sedimentation proved to be an effective isolation step that might be easily automatized and microwave assisted particle synthesis can be combined with continuous flow reactors, large scale automated peptide synthesis on colloidal supports can be foreseen.

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