Superparamagnetic γ-Fe_2O_3 nanoparticles with tailored functionality for protein separation†

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Polymer coated superparamagnetic γ-Fe_2O_3 nanoparticles were derivatized with a synthetic double-stranded RNA [poly(IC)], a known allosteric activator of the latent (2-5)A synthetase, to separate a single 35 kDa protein from a crude extract which cross reacted with antibodies raised against the sponge enzyme.

There is an ever-increasing demand for identifying or evolving new industrial enzymes capable of catalyzing processes under a range of different conditions. Examples include enzymes that are stable and active over long periods of time, those that are active in non-aqueous solvents, or that can accept and catalyze efficient turnover of various unnatural substrates. The identification and "rational" re-design of these proteins require a separation of these proteins. Magnetic nanoparticles would be ideally suited for protein separation in a "positive" sense where the magnetic beads bind in a highly selective fashion to an analyte target. In favourable cases, this would allow separation of the particle-bound proteins by magnetic decantation using a permanent magnet. To date, magnetic particles have been used primarily to separate and concentrate analytes for off-line detection in a non-specific manner.[1a–j] This paper describes a protocol to use and also to recycle nanoparticles specifically functionalized for protein separation.

As an example we chose a protein from the fresh water sponge *Lubomirskia baicalensis*. Most of the molecular and functional studies with proteins from sponges, in general and with immune molecules in particular, have been performed with the demosponges *Suberites domuncula*, *Geodia cydonium*,[2] and recently also with *Lubomirskia baicalensis*. Many of the immune-related molecules identified in sponges reveal high sequence and functional similarity with sequences found in the *Deuterostomia* taxon. Among them are (i) receptors, displaying immunoglobulin (Ig) domains with a high sequence similarity to the human Ig variable region,[3a] (ii) cytokines and (iii) interferon (IFN)-inducible antiviral proteins such as the 2′-5′-oligoadenylate synthetase [(2-5)A synthetase].[3b] The IFNs have been found so far only in vertebrates displaying antiviral, cell growth regulatory and immunomodulatory activity.[4]

Among the more than 100 genes whose expression levels are under the control of IFN in mammals,[5] are also the (2-5)A synthetases. They form a family of enzymes which must bind double-stranded RNA [dsRNA] in order to form an active enzymatic complex. The key enzyme of the 2–5A pathway, 2-5 A oligoadenylate synthetase [(2-5)A synthetase: EC 2.7.7.-] converts cellular ATP to a series of short 2′,5′-linked oligoadenylates with a general formula pppA(2′p5′A)$_n$, $n \geqslant 1$.[6] They synthesize 2′-5′-oligoadenylates (2-5)A to bind a, likewise latent, endonuclease [the RNase L]. After activation of the RNase L by 2-5A, the protein biosynthesis in viral infected cells will be blocked by a degradation of cellular and viral RNA.[7]

Recently it was demonstrated[8] that the demosponge *Geodia cydonium* comprises high levels of (2-5)A synthetase and produces larger amounts of (2-5)A oligomers ranging from dimers to octamers, with the accumulation of the dimer in a predominant proportion during the first stage of the reaction[14] with a level of synthesis higher than in mammalian cells. Until now, the enzyme could not be purified; moreover, the enzyme in the enriched fraction could not be stimulated by dsRNA but when immobilized into a poly(IC) membrane it has a high (2-5)A synthetase activity.

After cloning the enzyme from *G. cydonium*[9a] and later from *S. domuncula*,[9b] the assumed characteristic domains present in the deduced proteins, *e.g.* (i) the (2-5)A synthetase signature-1 and signature-2,[10] (ii) the ATP-binding site, which is essential for the enzyme activity,[11a,b] and (iii) the assumed dsRNA binding region,[11a] could be identified. Further experimental studies revealed that the sponge (2-5)A synthetase bacterial can be induced/activated in cells exposed to bacteria or to bacterial endotoxin lipopolysaccharide (LPS).[9]

In this contribution, we present a protocol for the extraction and purification of sponge (2-5)A synthetase from *Lubomirskia baicalensis* by making use of the binding affinity between this protein and dsRNA [poly(IC)], which had been immobilized onto γ-Fe_2O_3 nanocrystals (Chart 1). After the separation, proteins can be detached from the functionalized nanocrystals with urea and the magnetic beads can be recovered and recycled for further use. The liquid phase containing the (2-5)A synthetase undergoes conventional biochemical analysis. These reaction steps are summarized in Chart 1. After successful identification and purification, we cloned the *L. baicalensis* (2-5)A synthetase, raised antibodies against the recombinant protein to verify the enzyme and determined the expression level by *in situ* hybridization. Finally, animals were treated with poly(IC) to demonstrate that in

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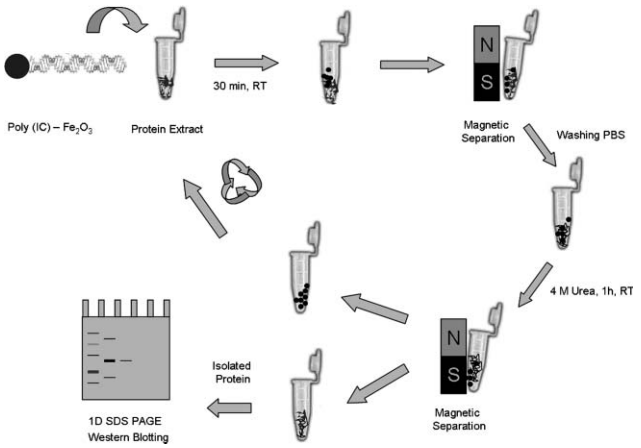

Chart 1 Isolation of (2-5)A synthetase from sponge extract using dsRNA [poly(IC)] functionalized superparamagnetic γ-Fe_2O_3 nanoparticles.

response to dsRNA they react with an increased expression of the *(2-5)A synthetase* gene.

Superparamagnetic γ-Fe_2O_3 nanoparticles were synthesized by the co-precipitation of ferrous and ferric ions in sodium hydroxide solution, as reported elsewhere.[12] Phase identification of the naked iron oxide nanoparticles was carried out using Mössbauer spectroscopy and magnetic susceptibility measurements.[13] Maghemite is superparamagnetic at ambient temperature for particle sizes smaller than 9 nm.

The γ-Fe_2O_3 nanoparticles were functionalized using a multidentate functional copolymer (M_n: 29.7 kg mol^{-1}, M_w = 58.5 kg mol^{-1}, 246 repeat units)[14] carrying catecholate groups as surface binding ligands for the iron oxide nanoparticles and free amino groups for the attachment of the poly(IC) ligands (Fig. 1). To remove unbound polymer, the coated magnetic particles in the solution were extracted using a magnetic particle concentrator (Dynal MPC1-50, Dynal Biotech, France) at room temperature. The isolated magnetic nanoparticles were washed with DMF, ensuring the removal of unreacted polymer, and subsequently dispersed in methyl imidazole buffer (MeIm, 0.1 M, pH 7.5). A portion of the washed magnetic particles was freeze-dried for subsequent characterization.

The presence of primary amine groups in the polymer ligand on the surface of the iron oxide nanoparticles permits the attachment of biomolecules through the phosphoramidite reaction.[13] The activated poly(IC) mixture was coupled to the amine functionalized iron oxide nanoparticles. The aliquots were immediately frozen. All experiments were carried out in RNase-free solutions and environment.

The average crystallite size of particles with and without functional polymer coating was estimated using transmission electron microscopy (TEM) using a Philips 420 instrument with an acceleration voltage of 120 kV. Fig. 2a shows the TEM image of the unfunctionalized γ-Fe_2O_3 nanoparticles with an average particle size of $\sim$12 nm. Fig. 2b shows a high resolution image of a γ-Fe_2O_3 particle. A TEM image of polymer functionalized particles is given in the ESI.†

The *Lubomirskia baicalensis* (2-5)A synthetase was bounded to the functionalized γ-Fe_2O_3 nanoparticles by incubation of sponge extract with poly(IC)-conjugated iron oxide nanoparticles for 30 minutes to allow binding of poly(IC) interacting proteins. The samples were then submitted to a magnetic field to concentrate the magnetic nanoparticles and washed twice with PBS buffer (phosphate-buffered saline), making use of the magnetic properties of the iron oxide nanoparticles. To remove the interacting protein, the samples were treated with 4 M urea (pH 7.0) for 30 minutes.

The supernatant was collected and analyzed by $NaDodSO_4$-PAGE (Fig. 3 bottom). After electrophoretic separation and conventional staining, a clear band with a size of 35 kDa could be visualized (Fig. 3 A lane b). This molecular weight corresponds to the calculated size of the (2-5)A synthetase, deduced from the cloned *Lubomirskia baicalensis* (2-5)A synthetase cDNA [35 748 kDa].

In order to verify that (i) the separated 35 kDa polypeptide represents the (2-5)A synthetase and (ii) the detachment of the proteins from the cluster proceeds by cleavage of the phosphate ester bond, polyclonal antibodies (anti-2-5A 322, anti-goat anti-rabbit IgG) which had been raised against the recombinant sponge protein were applied for western immunoblotting. Using this probe a clear cross-reactivity with the 35 kDa protein could be demonstrated (Fig. 3, bottom, B lane a). As a control PoAb-25AS were adsorbed with recombinant sponge (2-5)A synthetase. This antibody preparation did not show any significant cross-reactivity to a protein on the blot (Fig. 3, bottom B lane b). The influence of 4 M urea on the dsRNA-functionalized nanoparticles was studied in a time course experiment (Fig. 3, top). The samples were loaded onto 1% agarose gel. Incubation of poly(IC) functionalized γ-Fe_2O_3 nanoparticles at various time intervals (Fig. 3, top: lanes 1–5) with 4 M urea does not harm the phosphoramidite bond (Fig. 3, top: lanes 6–10).

In this work, we have prepared high capacity magnetic beads for protein separation by a reduction in particle size to provide a sufficiently large surface area and by superparamagnetic properties

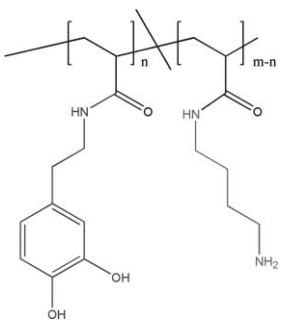

Fig. 1 Multidentate functional copolymer carrying dopamine anchor ligands for the surface binding and 1,3-butyldiamine ligands for the poly(IC) binding.

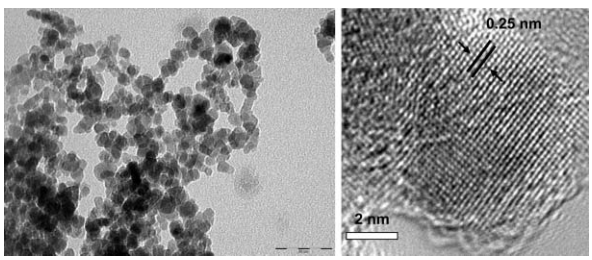

Fig. 2 General view of the ferromagnetic iron oxide sample by TEM (a); HRTEM picture of a particle displaying lattice fringes with *d*-spacings of 0.25 nm, which is characteristics of the (311) of maghemite structure (b).

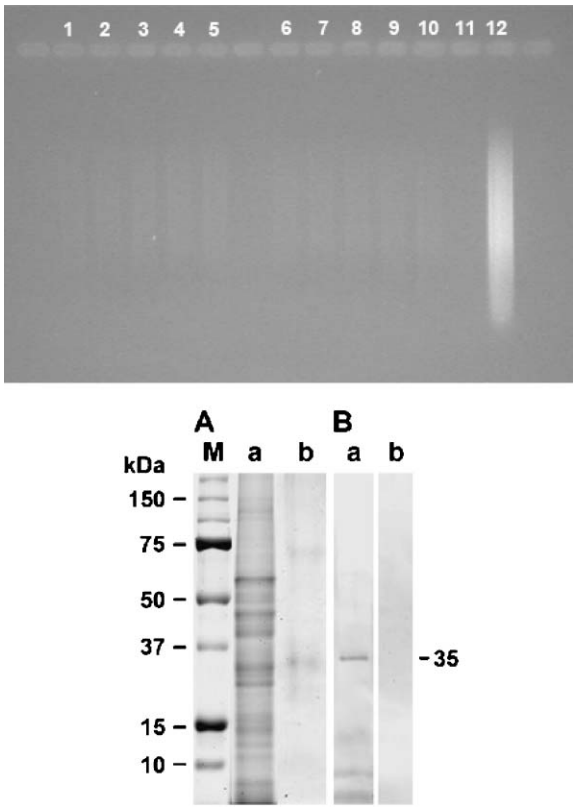

Fig. 3 Top: 1% agarose gel in TBE buffer, stained with EtBr for 30 min. Functionalized γ-Fe_2O_3 + EDC + poly(IC) incubated for 0 min (1), 30 min (2), 1 h (3), 2 h (4) and 3 h (5). Incubation of 1, 2, 3, 4 and 5 in the presence of 4 M urea for 0 min (6), 30 min (7), 1 h (8), 2 h (9) and 3 h (10). Controls were performed using functionalized γ-Fe_2O_3 nanoparticles (11) and poly(IC) (12). The photo was achieved using trans-Iluminator (BIO-RAD) connected to a PC and software Quantity One® version 4.2.2 (BIO-RAD). Bottom: isolation and identification of *L. baicalensis* (2-5)A synthetase. (**A**) Isolation of the (2-5)A synthetase by NaDodSO$_4$-PAGE. (Lane a) A crude extract was prepared and separated by gel electrophoresis. (Lane b) The crude extract was supplemented with poly(IC)–iron oxide conjugated nanoparticles, and after separation of the particles in a magnetic field and the following elution of the associated proteins, the soluble molecules were size separated. The gels were stained with Coomassie brilliant blue. M: a size marker was run in parallel. (**B**) Western blot analysis of the fraction, obtained after separation with poly(IC)–iron oxide conjugated nanoparticles. This fraction was subjected to NaDodSO$_4$-PAGE, followed by a transfer of the proteins to a membrane. This membrane was finally reacted with PoAb-25AS antibodies. The immunocomplexes were identified with labeled secondary antibodies. The 35 kDa protein can be identified in the poly(IC)–iron oxide nanoparticle fraction, after desorption with urea; (lane a). In a separate western blotting experiment, the membranes were reacted with PoAb-25AS which had been adsorbed with recombinant (2-5)A synthetase [ads]; no signal is seen (lane b).

of maghemite nanoparticles. Secondly, we have introduced a multidentate polymer with a tailored poly(IC) surface functionability for efficient coupling of the marine sponge (2-5)A synthetase to the beads. Using this surface bound polymer as a probe, a 35 kDa protein could be isolated from the crude extract. We have made use of the fact that sponge (2-5)A synthetase has the ability to bind to dsRNA. This hypothesis was based on a recent report by Wiens *et al.*[15] on the existence of an innate response system including the Toll-like T3 receptor, *i.e.* the identified (potential) antiviral defence system from sponges involves the (2-5)A synthetase. The product of this enzyme is (2-5)A which activates the latent RNase L which, in turn, degrades both viral and dsRNA cellular RNA. It was unknown, so far, whether the sponge (2-5)A synthetase has the ability to bind to dsRNA, a process which is required for conversion of the latent to the active form.

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