Spider Silk Capsules as Protective Reaction Containers for Enzymes

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Spider silk fibres are well known for their high tensile strength in combination with high elasticity. Based on the possibility of recombinant production of spider silk proteins, technical applications of spider silk materials are nowadays feasible. The engineered recombinant spider silk protein eADF4(C16) is based on the sequence of ADF4 (*Araneus diadematus* fibroin), one out of at least three proteins of the dragline silk of the European garden spider *A. diadematus*. The protein eADF4(C16) can be processed into different morphologies. Here, capsules of eADF4(C16) are assembled at an oil/water interface. These microcapsules are mechanically stable and can be used as a transport system for higher molecular weight compounds such as enzymes or chemical catalysts. Further, they can be regarded as a small enclosed reaction chamber with a semi-permeable membrane. Reactions can be initiated by diffusion of the reactants through the silk membrane. The eADF4(C16) capsules protect the enzyme β-galactosidase, used as model, against proteolysis. Functional α-complementation of β-galactosidase visualizes the controllable activation of an enzyme within such spider silk capsule, highlighting the broad applicability thereof as reaction containers, e.g., for enzymes.

1. Introduction

Encapsulation of enzymes for transport or protection, for example, against proteases, as well as for supporting their penetration into cells is a common theme.[1] Previously, polymer or protein microcapsules,[2–4] lipid vesicles, multilayered microcapsules,[5] lipid nanocapsules, polyelectrolyte capsules[6] or inorganic/organic nanocomposite microcapsules[7] have been employed for encapsulation of active substances. In some cases the capsule is formed around a solid core which has to be removed afterwards. Alternatively, the capsule membrane is tight, and release of the content is accomplished upon destruction of the capsule. In other cases the membrane is semi-permeable. Permeability could be triggered by a change of pH or ionic strength to allow selective diffusion of molecules through the membrane.[8]

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Different polymers have been used as encapsulation systems for drugs or enzymes, like proteins, collagens or alginates as examples for natural polymers, or polyethylene glycol (PEG) or polylactide (PLA) as examples for synthetic polymers.[9–14] Layer by layer (LbL) deposition of *Bombyx mori* silk fibroin on silica particles has been also used for formation of highly permeable microcapsules after dissolution of the inorganic core.[15–17] Silk proteins combine several features beneficial for their use in medical or pharmaceutical applications. For instance, they are biodegradable, biocompatible and they cause no immune response.[10] Furthermore, spider silk proteins can be nowadays produced in biotechnological processes ensuring a constant quality and preventing possible viral contaminations.[18] The recombinant spider silk protein eADF4(C16) can be processed into a number of different morphologies, most of them in all aqueous processes.[3,19–23]

Previously, we have shown that the spider silk protein eADF4(C16), based on the partially sequenced dragline silk protein ADF4 from *A. diadematus*,[24,25] self-assembles at a toluene/water interface to form a capsule.[3,26] eADF4(C16) capsules have a high β-sheet content, and despite their 50–70 nm thin shell they are mechanically stable as reflected by the Young's modulus of 0.7–3.6 GPa, which is comparable to the Young's modulus of capsids of a bacteriophage infecting *Bacillus subtilis*.[27] Further, they are chemically stable in presence of, e.g., 2% sodium dodecyl sulphate (SDS) and 8 M urea. The average molecular weight cut-off of 27 kDa makes these capsules suitable to encapsulate high molecular weight substances.[3,26]

Here, we tested eADF4(C16) capsules as reaction containers to protect enzymes against proteolysis and to control their activity. As a model enzyme we used β-galactosidase and employed its α-complementation to investigate whether a controlled enzyme activation could be accomplished within the capsules. In order to avoid the use of toxic toluene (as employed previously to produce spider silk capsules),[3] we established a new technique of capsule formation using medical grade silicon oil. Due to a slightly different silk structure formation at the silicon oil/water interface, an additional processing step had to be introduced to structurally stabilize the silk capsules.

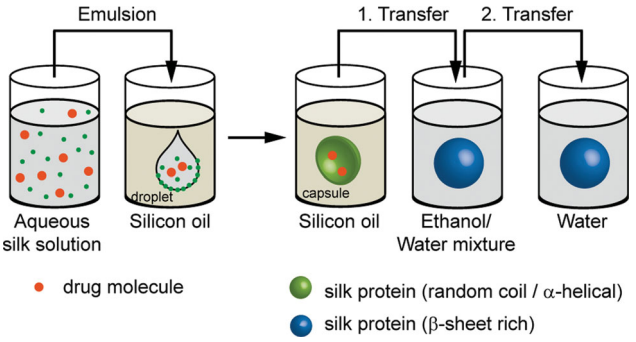

Figure 1. Silk capsule formation. An aqueous silk solution with encapsulate is emulsified in silicon oil. The amphiphilic eADF4(C16) protein localizes at the water/oil interface by diffusion. Upon self-assembly into a nanometer-thin but stable film it can be transferred into an aqueous phase by re-suspension in a water/ethanol mixture inducing β-sheet formation of the silk proteins, necessary to stabilize the silk structure. The final capsules can be transferred into water/buffered solutions.

2. Results and Discussion

2.1. Preparation of Spider Silk Capsules

In a previously established process to produce capsules an aqueous eADF4(C16) solution was emulsified in toluene.[3] Due to the amphiphilicity of eADF4(C16) it accumulates at the water/toluene interphase driven by diffusion. It could be shown that eADF4(C16) which is intrinsically unstructured in aqueous buffer adopted a β-sheet rich structure at the water/toluene interphase, yielding a stable film surrounding the aqueous droplet representing a capsule. One drawback of using toluene as the organic phase is its toxicity, which could impact especially biomedical applications. Here, we wanted to use silicone oil which in contrast to toluene is non-toxic, but does not induce a β-sheet structure like toluene does. In order to structurally stabilize the eADF4(C16) capsules, we therefore performed an additional processing step using ethanol to induce the β-sheet formation of eADF4(C16) (**Figure 1**), similar to the post-treatment of silk films on solid substrates.[20,22,28] Dimensions (1–30 µm), mechanical stability (0.7–3.0 GPa) and the molecular weight cut-off of the capsules were comparable to that of silk capsules produced with the toluene process (**Figure 2**).[23] By encapsulating FITC-labelled dextran we could further show that molecules with a molecular weight higher than 40 kDa remain inside the silk capsule, while smaller molecules diffuse out of the capsules after the transfer into water. The reported hydrodynamic diameter for 40 kDa FITC-Dextran is 4.3 nm,[29] which can be taken as a measure for the limit of the maximum size of the pores of eADF4(C16) capsules. The porosity of the eADF4(C16) capsules is therefore lower than that for capsules made of silkworm fibroin made by a layer-by-layer (LbL) technique. The porosity of LbL fibroin microcapsules with a shell thickness of 54 nm (similar to those of the eADF4(C16) capsules) was reported to be around 7 nm.[15] The lower porosity of the spider silk capsules is likely due to a higher crystallinity of the proteins.

Taken together, the lower porosity of the eADF4(C16) capsules along with the easier processing of spider silk solutions (a two-step process versus a multi-step process for *B. mori* fibroin) reflect an excellent basis for the highly efficient encapsulation of enzymes in comparison to the previously described LbL silk fibroin capsules.

2.2. β-Galactosidase Activity and α-Complementation

To confirm the usefulness of spider silk capsules to encapsulate active substances we used β-galactosidase as a model enzyme. β-galactosidase is a homo-tetrameric enzyme with a monomeric molecular weight of 116 kDa.[30] β-galactosidase activity can be spectroscopically determined using ONPG (ortho-nitrophenyl-β-D-galactopyranosid) (colorless) as a substrate: β-galactosidase

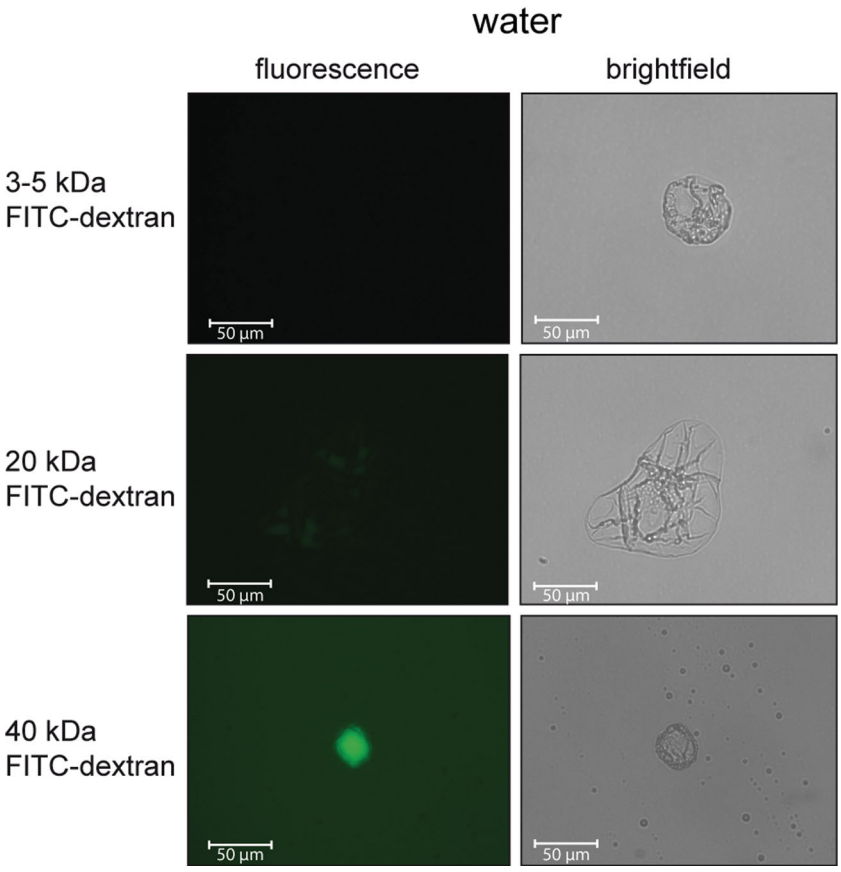

Figure 2. Molecular weight cut-off of eADF4(C16) capsules processed by using silicon oil as organic phase. Encapsulated FITC-labeled dextran with a molecular weight of 20 kDa or below diffuses out of the capsule, higher molecular weight encapsulates are retained within the capsule.

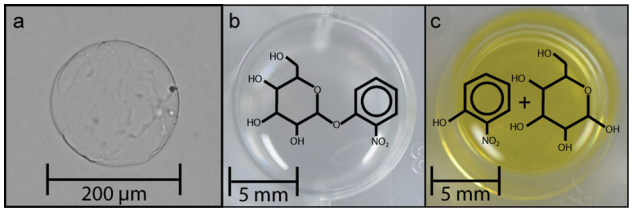

Figure 3. a) β-Galactosidase encapsulated within eADF4(C16) capsules. b) β-Galactosidase activity can be spectroscopically analyzed using ONPG (colorless) as a substrate. β-Galactosidase processes ONPG into c) o-nitrophenol (yellow) and galactose.

processes this substrate into galactose and o-nitrophenol, a yellow product that can be easily visualized and spectroscopically quantified (**Figure 3**).

Furthermore, based on the enzymatic properties of β-galactosidase, a system is available that can be activated on demand: α-complementation is a feature of β-galactosidase widely used e.g. in molecular biology. α-complementation reflects the activation of an inactive (dimeric) form of β-galactosidase (EA22 or α-acceptor) in the presence of the peptide ED28 (α-donor). The β-galactosidase fragment EA22 lacks amino terminal residues resulting in formation of an enzymatically inactive dimer.[31–33] Upon binding of the peptide ED28, dimerization of dimers is induced to form active tetramers.[32] This α-complemented tetramer has an equivalent substrate affinity in comparison to the wild-type enzyme. Here, we intended to selectively activate EA22 within the silk capsules by addition of ED28 to the surrounding media.

To analyse the general influence of eADF4(C16) on the enzyme activity, enzyme kinetics of β-galactosidase in the presence and absence of soluble eADF4(C16) were analysed for both β-galactosidase and the α-complemented enzyme (**Figure 4**). The presence of eADF4(C16) had no apparent influence on native β-galactosidase activity (Figure 4a,b, see also **Table 1**), but the activity of α-complemented enzyme was slightly increased in presence of eADF4(C16) (Figure 4c,d, Table 1), especially at 40 mU/mL. This observation could be due to stabilization effects of eADF4(C16) on either the α-acceptor or the α-donor (or both) as detected previously for other additives,[34,35] but it could as well be due to unspecific effects.

2.3. Activity and Protection of β-Galactosidase after Encapsulation

After encapsulation of β-galactosidase, ONPG was added to the medium, and the formation of o-nitrophenol was spectroscopically monitored over one hour of incubation (**Figure 5**a), indicating that both substrates and products could diffuse through the silk membrane. Even α-complementation of the non-active β-galactosidase fragment EA22 was possible by adding ED28 to the buffer surrounding the capsules, indicating that activation of inactive enzymes encapsulated in eADF4(C16) can be obtained using external triggers with molecular weights

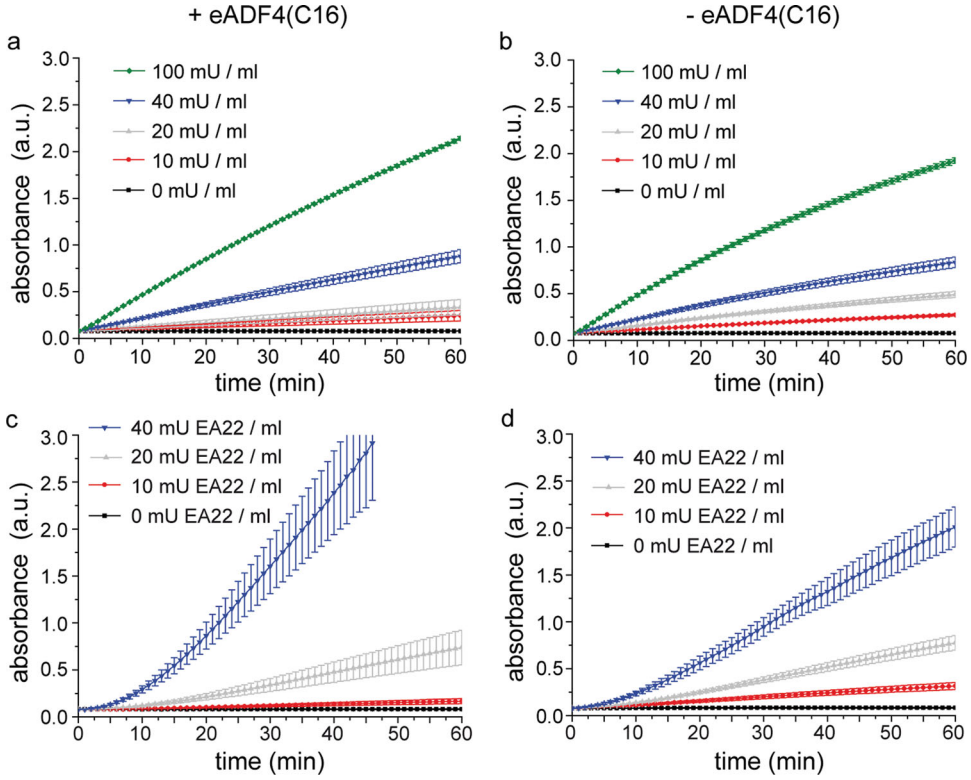

Figure 4. Activity of β-galactosidase (a,b) and reactivated β-galactosidase (EA22) after α-complementation (c,d) measured by o-nitrophenol absorbance in the presence (a,c) and in the absence (b,d) of 0.8 µM eADF4(C16) (soluble), in order to evaluate the general influence of the spider silk protein on the enzyme's activity.

Table 1. Turnover rate of β-galactosidase in absence and presence of 0.8 µM soluble eADF4(C16) in ng/min. n.d.: not determined.

	β-galactosidase + eADF4(C16)	β-galactosidase without eADF4(C16)	α-complementation + eADF4(C16)	α-complementation without eADF4(C16)
100 mU/mL	10.0 ± 0.1	8.9 ± 0.1	n.d.	n.d.
40 mU/mL	3.9 ± 0.3	3.7 ± 0.3	21.1 ± 3.8	10.6 ± 1.2
20 mU/mL	1.2 ± 0.4	2.0 ± 0.2	3.7 ± 1.1	3.6 ± 0.4
10 mU/mL	0.8 ± 0.3	0.9 ± 0.1	0.5 ± 0.1	1.1 ± 0.1

below 27 kDa (Figure 5b). The measured activity of encapsulated, α-complemented β-galactosidase versus that of wild type β-galactosidase was slightly lower, probably due to a lower availability of the α-donor within the capsule. Since entering the inner volume of the capsule is driven by diffusion across the capsule membrane, unspecific interactions between the α-donor and the silk proteins are feasible which will limit free diffusion of the α-donor and which will significantly influence its concentration in the capsule interior.

Next, we examined if silk capsules can protect encapsulated β-galactosidase against proteases. Importantly, such protection is only possible against proteases that do not degrade the spider silk capsules. However, the list of silk-active proteases is short and biodegradation is typically slow.[36] We exemplarily incubated the capsules with endoproteinase AspN, known to specifically digest β-galactosidase at 64 positions[37] (**Table 2**), but not eADF4(C16). Further, AspN has a molecular weight of 27 kDa which is right at the molecular weight cut-off of the silk capsules. As expected, encapsulated β-galactosidase was well-protected within the spider silk capsule and showed indistinguishable activity in the absence or presence of AspN (**Figure 6**a,b). Non-encapsulated β-galactosidase, however, was inactivated in the presence of AspN (Figure 6c) after four hours of incubation. Importantly, empty eADF4(C16) capsules did not stabilize β-galactosidase (in the surrounding solution) against proteolysis (data not shown).

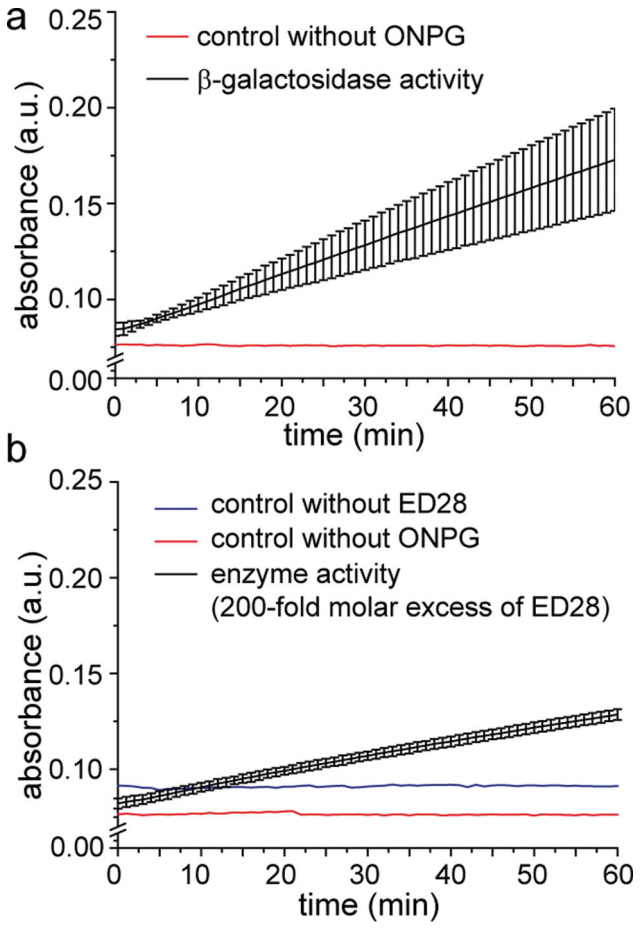

Figure 5. Activity of encapsulated β-galactosidase (a) and α-complemented EA22 (b) measured by o-nitrophenol absorbance.

3. Conclusion

We could show that eADF4(C16) silk capsules can be produced using (medical grade) silicon oil instead of toxic toluene as an organic phase. However, to stabilize the silk capsules (i.e., the underlying protein structure) an additional processing step was necessary using a water/ethanol bath. We could further show that eADF4(C16) capsules can be used to encapsulate active enzymes, as well as inactive enzyme precursors/intermediates which can be activated on demand. Here, we used β-galactosidase as a well-described model system to show the full potential of the spider silk capsule system. The encapsulated enzymes stay active even in the presence of site-specific proteases in the surrounding medium, and enzyme precursors/intermediates can be activated inside the capsules by external triggers (**Figure 7**).

Due to the previously limited availability of engineered spider silk, most of the silk studies on biomaterials applications have been carried out with *B. mori* silk fibroin in the past.[36,38,39] However, similar applications can be envisioned for

Table 2. AspN cleavage sites of β-galactosidase calculated using *ExPASy*.

Protease	No. of cleavage sites	Amino acid number after which the enzyme cleaves
Endoproteinase AspN	64	5 15 45 77 82 96 130 144 164 172 193 199 201 211 224 233 234 252 280 287 319 329 368 375 403 411 428 429 447 469 479 492 497 507 509 569 572 579 591 594 598 610 648 659 671 746 772 782 790 792 802 828 832 859 869 875 908 916 919 924 954 987 996 997

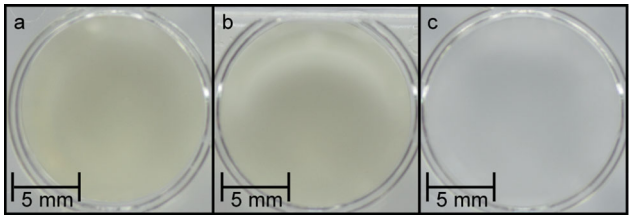

Figure 6. β-Galactosidase activity in presence of AspN visualized by o-nitrophenol absorbance. a) Positive control (encapsulated β-galactosidase, no AspN). b) Encapsulated β-galactosidase in presence of AspN. c) Negative control (not encapsulated β-galactosidase, in presence of AspN).

recombinant spider silk proteins due to the similarity in their physicochemical properties. Engineered spider silk proteins might be preferable due to the potential of silk protein design according to specific requirements and eventually improved mechanical properties. One advantage of recombinant spider silk proteins is the ease of genetic modification which allows the direct incorporation of functional groups into the silk proteins.[38,40] The recombinant spider silk capsules are therefore suitable to be used as tunable, protective enzyme reaction containers for certain technical as well as medical applications.

4. Experimental Section

Capsule Preparation: The engineered spider silk protein eADF4(C16) was produced and purified as described previously.[20] eADF4(C16) was dissolved in 6 M GdmSCN and dialysed against buffer (10 mM Tris/HCl, pH 8) overnight. 5 µL of aqueous protein solution with a concentration of approx. 5 mg/mL (+/– β-galactosidase) were re-suspended in 500 µL silicon oil M100 (Roth, Karlsruhe, Germany) by using a vortex mixer for 45 s. The freshly assembled capsules were transferred into a water/ethanol mixture to induce β-sheet formation, followed by three washing steps with water or buffer.

Enzyme Kinetics: β-Galactosidase activity was monitored using different enzyme concentrations with 1.33 µM ONPG as a substrate. Processed o-nitrophenol was quantified (absorption, 420 nm, 37 °C) using an UV–vis Spectrophotometer (Cary 50 Bio, Varian, Darmstadt, Germany). Enzyme kinetics were monitored in the presence and absence of 0.8 µM soluble eADF4(C16). β-Galactosidase was purchased from Sigma-Aldrich (Germany). α-complementation enzyme and peptide were provided by Roche Diagnostics.

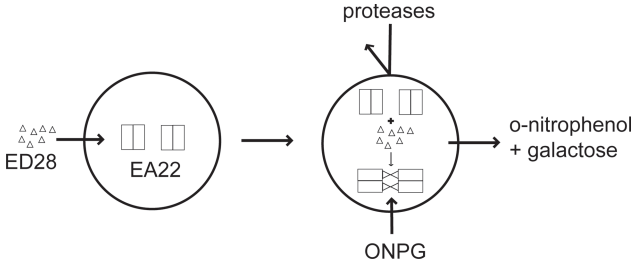

Figure 7. Silk capsules reflect semi-permeable reaction containers. Encapsulated α-acceptor (EA22) is activated upon adding the α-complementation peptide (ED28) to the surrounding medium, which diffuses into the capsule. The encapsulated enzyme is protected against proteolysis.

Protease Digestion: Capsules containing β-galactosidase were incubated in buffer containing 160 ng/mL endoproteinase AspN (Roche Diagnostics GmbH, Mannheim, Germany) for 4 h at 37 °C. The digestion was stopped by adding buffer Z (100 mM sodium phosphate pH 7, 10 mM KCl, 1 mM magnesium sulphate and 50 mM β-mercaptoethanol), followed by addition of 1.33 µM ONPG to determine β-galactosidase activity. As a control, non-encapsulated β-galactosidase was incubated together with endoproteinase AspN and in the presence and in the absence of eADF4(C16) capsules.

Molecular Weight Cut-Off: 40 µg FITC-labelled dextran (3–5 kDa, 20 kDa or 40 kDa) was encapsulated in the dark, and capsules were analysed in silicon oil as well as water using a fluorescence microscope (Leica DMI3000 B, Leica, Wetzlar, Germany).

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