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Reversible pH Lability of Crosslinked Vault Nanocapsules

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Abstract

Vaults are ubiquitous, self-assembled protein nanocapsules with dimension in the sub- 100 nm range that are conserved across diverse phyla from worms to humans. Their normal presence in humans at a copy number of over 10,000/cell makes them attractive as potential drug delivery vehicles. Toward this goal, bifunctional amine-reactive reagents are shown to be useful for the reversible crosslinking of recombinant vaults such that they may be closed and opened in a controllable manner.

Introduction

The application of nanotechnology to medicine has emerged as an area of intense interest, particularly the creation of nanosystems for drug delivery. The incorporation of drug molecules into or on nanoparticles often results in improvement of drug pharmacokinetics and/or biodistribution. Free drugs normally do not self-target to specific tissue and therefore may cause undesirable side effects, while drug delivery vehicles often can be designed with surface ligands for targeted delivery. In addition, delivery systems also can make possible controlled release of drugs in order to maintain them at a near optimal therapeutic level.[1-3] Moreover, drug delivery systems can improve the transport properties and systemic half-life of therapeutics that normally are insoluble in the bloodstream or are otherwise unstable *in vivo*.4 The most widely explored drug delivery systems include lipid- or polymer-based nanoparticles, or nonviral-based vectors, such as micelles, liposomes, polymer-drug conjugates, and polymer microspheres.1, [2] Others have investigated the use of virus-like particles (VLPs), or viral-based vectors, which are composed of self-assembling viral coat proteins for the encapsulation of desired drugs or DNA.[5] However, both viral and nonviral-based vectors have encountered serious challenges to their development as the ideal drug/gene delivery vehicle. Limitations include drug or gene loading issues, controllability of drug release, ease of manufacture, targetability to disease sites, biocompatibility and immunogenicity. In this work, we put forward vaults, a self-assembling protein nanocapsule found in normal human cells, as a potential drug or gene delivery system. Vault particles possess many features making them very promising vehicles for the delivery of therapeutic

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agents including self-assembly, ~100 nm size range, emerging atomic-level structural information, recombinant production system, existing features for targeting species to the large lumen (~ 5×10^4 nm^3), and a dynamic structure that may be controlled for manipulation of drug release kinetics. Further, the natural presence of vaults in humans ensures their biocompatibility, and indications to date are that they are non-immunogenic. These attributes provide vaults with enormous potential as a drug/gene delivery platform.[2-4]

Vaults are ribonucleoprotein nanocapsules which are found to be highly conserved in most eukaryotic species from worms to humans. The native function of vaults is unknown, yet a recent study has suggested a role associated with resistance of lung infection in epithelial cells.[6] Native vaults have molecular weight of 13 MDa[7] and dimensions of 72.5 nm × 41 nm.[8] They have a hollow interior compartment with a volume of 5×10^4 nm^3, which is large enough to accommodate hundreds of proteins.[9] The large vault lumen provides excellent space for the reversible encapsulation of drugs or DNA. Vaults were found to “open” when deposited onto polylysine-coated mica surfaces. Freeze-etch electron microscopy with platinum shadowing was utilized to image such vaults, and they appeared to disassemble into two halves and each half expands into a flowerlike shape with eight rectangular petals.[7] The ability to assemble and then trigger the disassembly of vaults by some external stimulus could enable the effective loading and releasing of encapsulated materials.

Native vaults consist of multiple copies of three proteins and an untranslated RNA (vault RNA). The major vault protein (MVP) exists in a total of 96 copies per vault particle, and they make up more than 70% of the total vault mass. The other two vault proteins are vault poly-(ADP-ribose) polymerase (VPARP) and telomerase-associated protein 1 (TEP1).8 VPARP is known to bind to MVP in the vault lumen8 and a minimal fragment (M-INT) of VPARP that binds to MVP has been produced.3 M-INT has been demonstrated to be an effective shuttle for the selective loading of species in the vault lumen.3 VPARP and TEP1 do not appear to contribute to the overall vault shape since the expression of MVP alone can self-assemble into the distinctive cap and barrel structure (Figure 1a).10 Since each vault is composed of 96 copies of MVP, it is believed that 48 copies of MVP make up each half vault with 6 MVPs corresponding with each “flower petal.”8 A 9 Å draft crystal structure of vaults along with various cryoelectron microscopy (cryoEM) experiments using different functionalized vaults have been carried out to determine the location of the C- and N-termini of each MVP chain.8, 11 These studies indicate that the C-termini of all MVPs are located at the cap (48 on each cap), whereas the N-termini are all collected at the vault waist in the lumen confirming earlier studies using difference density mapping of cryoEM reconstruction images.8, 11

Recombinant vaults were synthesized using a baculovirus expression system in Sf9 insect cells.[10] Protein or peptide tags can be added to the N-terminus of vaults to create functionalized nanocapsules with, for example, specific binding affinities or enzymatic activities.[8] For the experiments conducted in this paper, CP-MVP vaults were utilized where each MVP has an N-terminus modified with a cysteine-rich, 12-amino acid peptide tag derived from the metallothionine protein.[8] CP-MVP vaults do not contain the minor vault proteins, and they have dimensions slightly different from native vaults of 73.7 nm × 41 nm. They are found to be the most stable vault constructs thus far produced with consistent size, shape, and conformation.[8, 11]

Previous studies have shown that vaults exhibit dynamic structural change in solution, which allows entry and exit of materials.[12] In fact, moderate size proteins can gain access into the vault interior over time.[12] In order to successfully utilize vaults as vehicles for encapsulating material, we have proposed the use of various crosslinking reagents to stabilize the vault structure. We established earlier that vault dissociation into halves is triggered at pH < 4.0[13]

thus vault pH stability may be used to assess crosslinking effectiveness. Our goal is to create reversibly crosslinked vaults for controlled drug or DNA entrapment and release.

Sulfhydryl-Reactive Crosslinking Reagents

In an effort to exploit the added cysteine residues present in the waist region (N-termini) of the CP-MVP vaults, three homobifunctional, sulfhydryl-reactive crosslinking reagents of varying length with maleimide functional groups were first investigated as means to crosslink vault particles. Bis-maleimidohexane14-16 (BMH) and bis-maleimidoethane (BMOE) were purchased from Pierce Biotechnology, Inc. (Rockford, IL), and MAL-PEG-MAL-3400 (molecular weight = 3400 Da) was obtained from Nektar Therapeutics (Huntsville, AL) (see Table 1). All crosslinkers were prepared initially as 20 mM stock solutions in DMSO. The desired amount of CP-MVP vaults were mixed with the specific crosslinker at a final concentration of 1 mM in 20 mM MES buffer, pH 6.5 (Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 4 °C overnight. After the reaction, the vaults were separated from excess crosslinkers by centrifugal filtration (Millipore, Microcon YM-30, 30,000 MWCO) at 10,000 rpm for 8-10 mins. The crosslinked vault samples were subsequently washed with 20 mM MES buffer (pH 6.5) by centrifugal filtration for 3 to 5 times.

SDS-PAGE was used to verify CP-MVP polypeptide crosslinking within vaults. Since SDS-PAGE is run under denaturing conditions, normal vault particles are expected to disintegrate into CP-MVP polypeptides of ~100 kD. When crosslinks are introduced between CP-MVPs, higher molecular weight products are expected. Figure 1 (b) illustrates the result of crosslinking reaction using all three of the sulfhydryl-reactive crosslinkers listed in Table 1. The disappearance of MVP monomer band and the presence of higher molecular weight bands after the crosslinking reaction indicated that a large proportion of the individual CP-MVP polypeptides in the vaults were covalently coupled as a result of the crosslinking reaction. The sizes of the higher molecular weight bands suggested a distribution of dimers, trimers, tetramers, and larger multimers were formed. The gel also confirmed that the washing step after the crosslinking reaction did not disrupt the linkage. However, SDS-PAGE does not provide insight into possible improvement of vault stability as a result of crosslinking.

In order to assess the stability of vaults crosslinked via available cysteine sulfhydryl groups, transmission electron microscopy (TEM) was utilized to compare crosslinked vaults at neutral pH and at pH 3.4. The TEM procedure used to examine vaults was described previously.[13] Figure 1 (c) and (d) show images of MAL-PEG-MAL-crosslinked CP-MVP vault samples treated at pH 6.5 (20 mM MES) and pH 3.4 (64 mM citrate phosphate), respectively. Vault particles in Figure 1 (c) retained the distinctive cap/barrel vault shape (compared to Figure 1a), suggesting that the presence of covalently attached crosslinkers did not alter overall vault shape at neutral pH. However, MAL-PEG-MAL-crosslinked vaults dissociated into half vault structures as shown in Figure 1 (d). This TEM result combined with the SDS-PAGE data suggested that sulfhydryl-reactive crosslinkers do not couple opposite vault halves, rather they introduce crosslinks within each individual vault half. Both BMH- and BMOE-crosslinked CP-MVP vaults exhibited similar behavior upon low pH treatment to MAL-PEG-MAL-crosslinked vaults (data not shown).

Amine-Reactive Crosslinking Reagents

Since sulfhydryl-reactive crosslinkers did not appear to crosslink vault halves, amine-reactive crosslinking reagents were investigated. Two homobifunctional, amine-reactive crosslinkers were utilized for this experiment: (1) EGS17, 18 [ethylene glycobis(succinimidylsuccinate)] which is non-cleavable, and (2) DSP [dithiobis

(succinimidylpropionate)] which is cleavable by reducing a disulfide bond present in the spacer arm (Pierce Biotechnology, Inc., Rockford, IL). These crosslinker structures are shown in Table 2. Both of these crosslinkers are based on NHS-ester chemistry,[17] which targets the ε-amines on lysine side chains as well as the α-amines on protein/peptide N-termini. Since all 96 N-termini of each MVP chain are located at the vault waist,[8] it was thought that amine-reactive crosslinkers directed toward the N-termini might lead to significant crosslinking between vault halves. In addition, a recently published, draft crystal structure of the vault particle suggested that the chosen amine-reactive crosslinkers should be capable of bridging the distance between the N-terminal amines of the vault halves.[11]

The reaction with amine-reactive crosslinking reagents was carried out in a similar manner to that with the sulfhydryl-reactive crosslinkers. Stocks of crosslinkers were prepared fresh at 20 or 50 mM in DMSO prior to addition to CP-MVP vault preparations (~ 1 mg/mL) at final crosslinker concentration of 1, 2, or 5 mM in 20 mM MES buffer, pH 6.5. The reaction was conducted at 4 °C overnight, followed by washing of the crosslinked sample using 30,000 MWCO centrifugal filters as described above. SDS-PAGE and TEM again were employed to assess vault crosslinking.

Figure 2 (a) shows the SDS-PAGE result for EGS-crosslinked CP-MVP vaults. As indicated by the shift of protein product to higher molecular weight bands after crosslinking, the coupling reaction apparently result in covalent links between CP-MVP polypeptides. These results were similar to those obtained with the sulfhydryl-reactive crosslinkers, except that more higher molecular weight crosslinked CP-MVP polypeptides were evident on the gel as the concentration of EGS was increased. These linkages between CP-MVP polypeptides also were stable during the washing step to remove any excess crosslinkers, as expected.

In order to determine if the amine-reactive crosslinkers caused substantial crosslinks between opposite vault halves, TEM was again utilized. Figure 2 (b) and (c) show TEM images of EGS-crosslinked CP-MVP vaults at pH 6.5 and 3.4 respectively. When vaults crosslinked with EGS were exposed to low pH, they remained intact as indicated by the very distinctive cap and barrel vault shape, suggesting that the two opposite halves are covalently coupled by the crosslinkers. However, the crosslinked vaults appeared to shrink in size at low pH, although the cause for the shrinkage is unknown. More importantly, the presence of EGS crosslinks does not affect the overall vault structure at pH 6.5, as seen by the similarity between non-covalently assembled CP-MVP vaults and crosslinked CP-MVP vaults at pH 6.5 in TEM images.[13]

In order to confirm findings based on TEM images, which provide data on a very limited vault sample, small-angle X-ray scattering (SAXS) was employed to gather structural data from a vault ensemble. SAXS provides the capability to gather structural information by obtaining the form factor of a particle from measurement of scattered light intensity of a diluted sample with randomly oriented particles in the X-ray beam.19-21 All SAXS studies were performed with synchrotron X-ray radiation on beamline 4-2 at the Stanford Synchrotron Radiation Laboratory. For each experiment, vaults at a concentration of 1.5 mg/mL in a total volume of 25 μL was prepared in a quartz capillary. Scattered X-rays were collected at a distance of 2.5 m (λ = 1.38 Å, 20 scans of 30 s each) on a MarCCD detector as 512 × 512 pixel images. The images were radially averaged to obtain one-dimensional scattering curves. The form factor, I(q), was obtained by subtracting from the background scattering caused by buffer alone. The observable q range was 0.006 to 0.25 $Å^{-1}$. The distance distribution function, P(r), was computed as the inverse Fourier transform of I(q) within the limits of r set by the q range.[22] The collected data were then analyzed using the ATSAS package.23

Figure 3 shows the P(r) scattering length density distributions for (a) noncovalently assembled CP-MVP vaults and (b) EGS-crosslinked CP-MVP vaults at pH 6.5 and 3.4, where P(r) is a distance distribution function representing the radially averaged probability of correlated electron density at a separation of r within the macromolecule. Macromolecules with different conformations and/or sizes are expected to exhibit different P(r) profiles.[20] In Figure 3 (a), a typical P(r) curve of CP-MVP vaults at pH 6.5 (solid line) shows an asymmetric curve representing the hollow nature of the vault shell. The peak at 350 Å corresponds to the diameter of the short axis of the vault particle modeled as an ellipsoidal shell. At pH 3.4, CP-MVP vaults have been shown to dissociate into half vaults, along with other structures such as opened half vaults, half vault pairs and aggregates.[13] Vaults treated at pH 3.4, therefore, give rise to a more heterogeneous population of structures, which results in destructive interference and a decrease in peak intensity as represented by the dotted line of Figure 3 (a). The more Gaussian-like distribution at pH 3.4 suggests a more spherical overall vault shape, which may represent the shape of a single half vault. The extra probability at larger radius compared to pH 6.5 indicates the presence of other non-compact structures such as opened half vaults, half vault pairs, and aggregates. This data collectively supports our previous observation of vault dissociation at low pH.[13]

In contrast, CP-MVP vaults crosslinked with EGS exhibit very different behavior as shown in Figure 3 (b). The two curves of EGS-crosslinked vaults at pH 6.5 (solid line) and 3.4 (dotted line) have similar peak intensity, suggesting that crosslinked vaults at pH 3.4 stay relatively homogeneous as shown in the TEM image (Figure 2c) dominating by intact vaults. A slightly shorter radius was measured for EGS-crosslinked vaults at pH 3.4, confirming crosslinked vaults shrinkage which was observed using TEM. In addition, the P(r) curve for pH 3.4 vaults is more Gaussian-like, implying that the shrunk vaults are more spherical than regular vaults at pH 6.5. The extra distribution at larger radius is no longer observed here, indicating an absence of opened half vaults and half vault pairs seen in the uncrosslinked vault sample.

CP-MVP vaults also were crosslinked with DSP, a cleavable, homobifunctional, amine-reactive coupling agent. The same crosslinking conditions were used with DSP as were used previously with EGS, and the SDS-PAGE result with DSP-crosslinked CP-MVP vaults appeared similar to those with EGS (Figure 4 a). After crosslinking, the DSP-treated vault sample was reacted with 25 mM DTT at 37 °C for 1 h in order to cleave the disulfide bridge in DSP. Figure 4 (a) lane D shows that a very small amount of crosslinked CP-MVP remained after chemical reduction. Figure 4 (b) to (e) illustrate the TEM images of DSP-crosslinked CP-MVP vaults before and after reduction by DTT at pH 6.5 and 3.4. Similar to the EGS-crosslinked CP-MVP vaults, the DSP-treated vaults appeared to remain intact after exposure to pH 3.4 as shown by the identifiable cap and barrel vault shape. However, more aggregation was observed in the DSP-crosslinked sample at pH 3.4. After reducing with 25 mM DTT for 1 h, the DSP-crosslinked vaults resume half vault structures at pH 3.4, implying that the disulfide bond present in the DSP was reduced therefore allowing vaults to dissociate. The most striking finding was that uncrosslinked, DSP-crosslinked and DSP-crosslinked and reduced vault structures appeared the same at pH 6.5. These results suggest that the overall vault structure stayed constant at pH 6.5, while structural stability can be reversibly controlled via the DSP crosslinker.

Conclusion

There is clearly a need for drug delivery systems that can be used to facilitate the release of drug molecules. The next generation of nanomedicine promises to employ delivery systems that will help target specific tissue, that will control the release of encapsulated materials therefore prolonging the half-life of the drug, and that will make possible the transport of

drugs which are normally insoluble in aqueous solution. The self-assembled protein nanocapsules called vaults appear to be an attractive drug delivery system due to their biocompatibility, the lack of immunogenic effect when taken up by mammalian cells,[3] and the ability to control their structural dynamics in solution. While vaults normally dissociate into halves at pH less than 4.0, covalent crosslinking of available amine groups renders vaults stable at low pH as evidenced by TEM. In contrast, covalent crosslinking of cysteine sulfhydryl groups, made available at the vault waist via an N-terminal tag, does not bestow pH stability on treated vaults. When a cleavable, amine-reactive bifunctional coupling reagent is used, crosslinked vault pH stability may be reversed by cleaving crosslinks.

These results suggest that vaults may be engineered for reversible encapsulation of materials. For example, vaults, which are natural residents of human cells, may be designed to carry drugs or genes prior to crosslinking, which will be delivered to a targeted site where the release of carrier molecule could be triggered by cleaving the vault crosslinks. In our companion paper titled “Encapsulation of Semiconducting Polymers Using Protein Vault Cages” by Ng *et al*., the loading of materials into nonconvalently assembled vaults and crosslinked vaults was demonstrated using a semiconducting polymer. Taken together our work illustrates the potential of vault nanocapules as drug or gene delivery vehicles.

Acknowledgments

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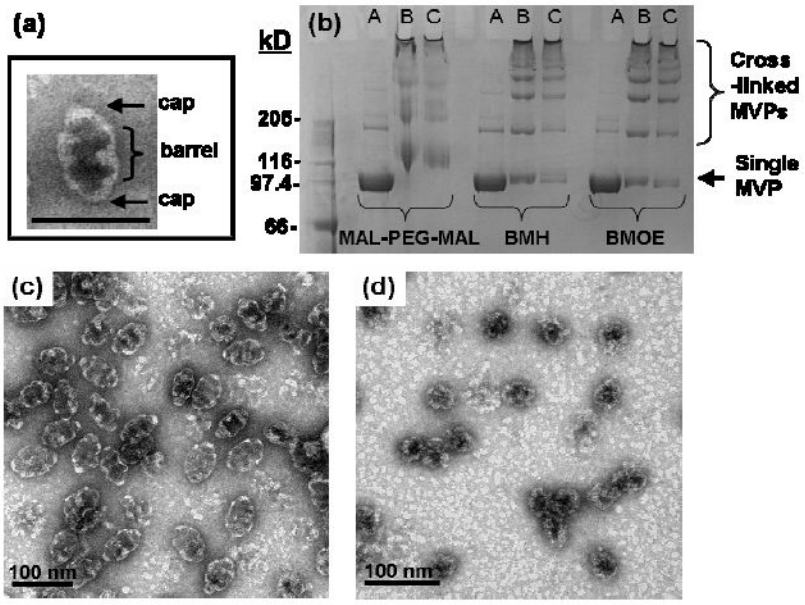


Figure 1.

CP-MVP vaults treated with sulfhydryl reactive crosslinkers resulted in covalently coupled CP-MVPs, but these vaults still dissociate into halves at low pH. (a) TEM image of an intact CP-MVP vault before covalent crosslinking. Scale bar represents 100 nm. (b) SDS-PAGE result for indicated sulfhydryl-reactive crosslinking experiments treated overnight at 4°C, stained with Coomassie brilliant blue. *Lanes A* represent CP-MVP vaults before crosslinking experiments; *lanes B* and *C* represent CP-MVP vaults after crosslinking before and after centrifugation, respectively, to remove excess crosslinkers. (c) TEM images of MAL-PEG-MAL-crosslinked CP-MVP vaults at pH 6.5. (d) TEM images of MAL-PEG-MAL-crosslinked CP-MVP vaults treated at pH 3.4 condition for 1 h.

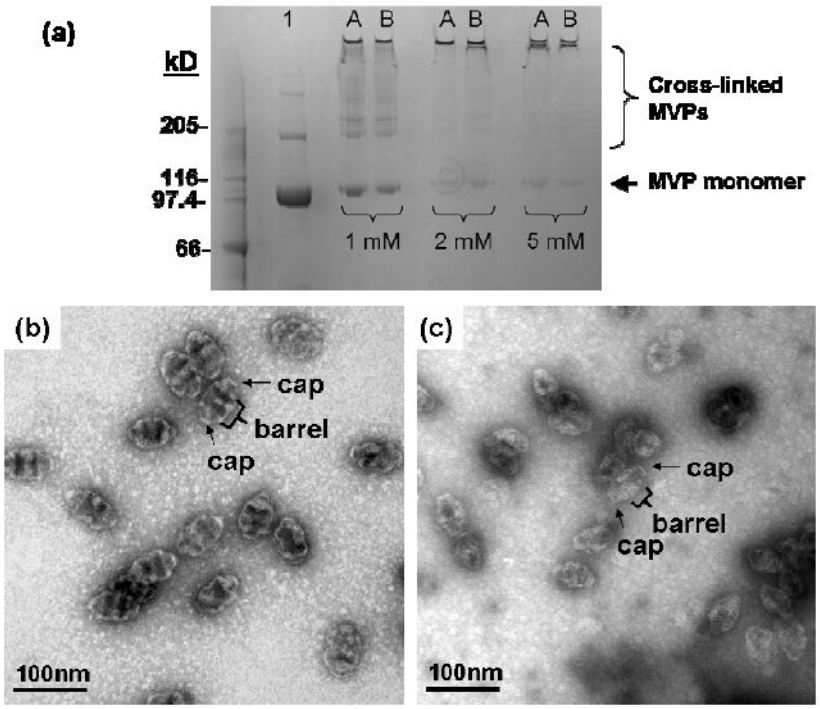


Figure 2.

EGS crosslinking of CP-MVP vaults resulted in stable vaults that stay intact at low pH. (a) SDS-PAGE result for EGS crosslinking experiments treated overnight at 4°C, stained with Coomassie brilliant blue. *Lane 1* represents CP-MVP vaults before crosslinking; *lanes A* and *B* represent CP-MVP vaults after crosslinking before and after centrifugation, respectively, to remove excess crosslinkers. (b) TEM image of EGS crosslinked CP-MVP vaults at pH 6.5. (c) TEM image of EGS crosslinked CP-MVP vaults treated at pH 3.4 for 1 h.

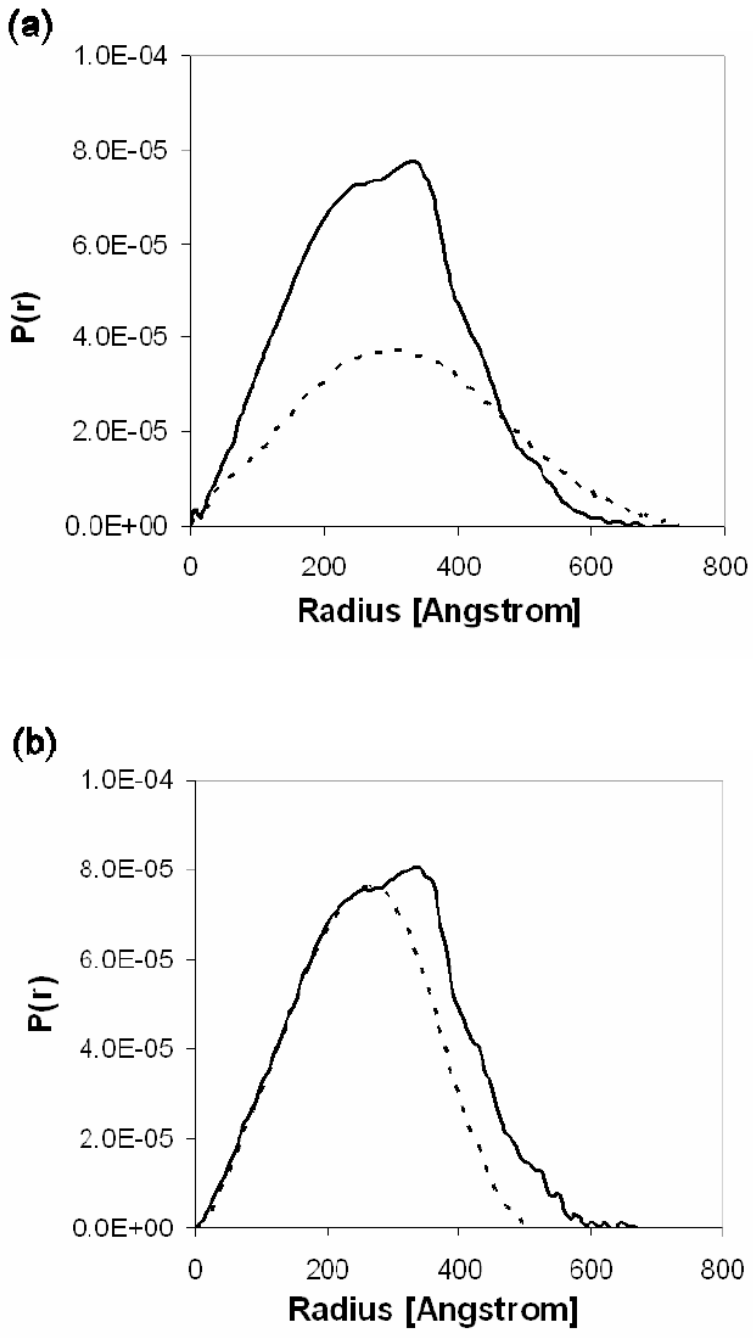


Figure 3.
Electron pair distribution function, P(r), of (a) regular, uncrosslinked CP-MVP vaults and (b) EGS-crosslinked CP-MVP vaults. Solid lines (—) represent sample at pH 6.5 and dotted lines (----) represent sample at pH 3.4.

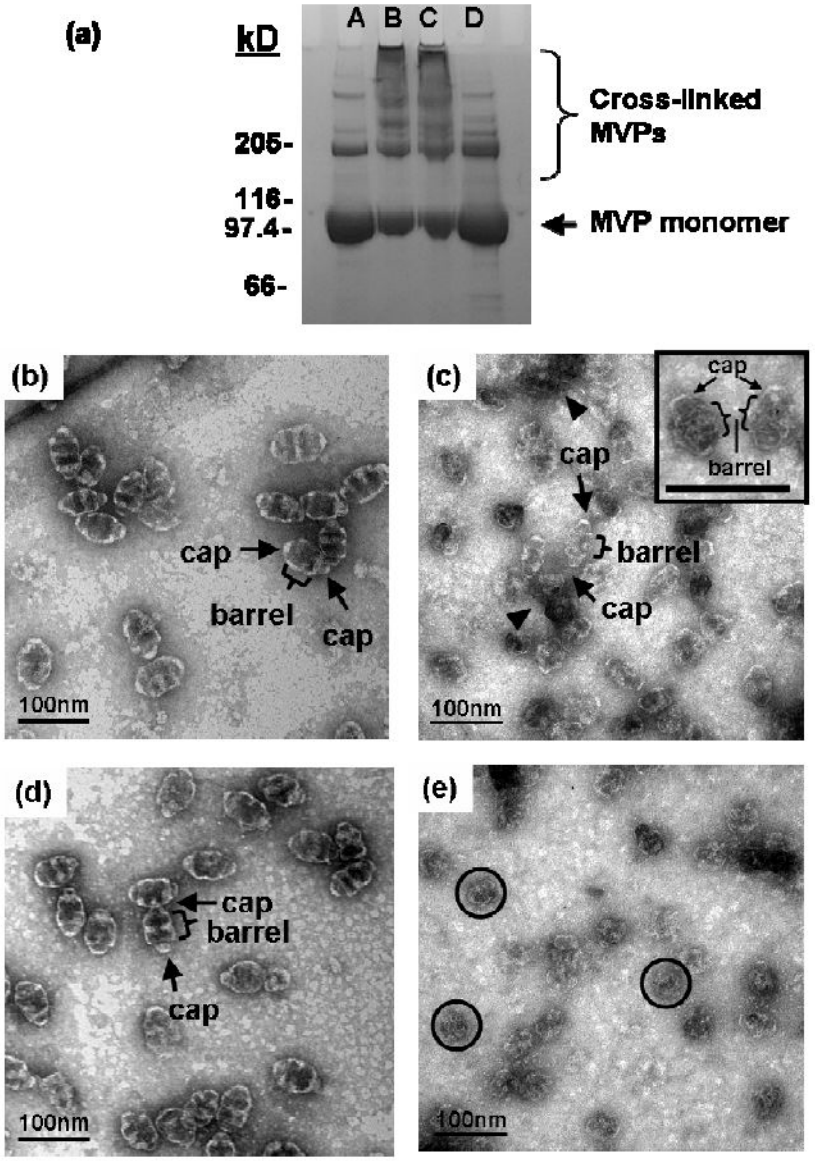


Figure 4.

DSP-crosslinked CP-MVP vaults remain intact at low pH, but become pH-labile after the crosslinker is cleaved by reduction with DTT. (a) SDS-PAGE result for DSP crosslinking experiments treated at 4°C overnight, stained with Coomassie brilliant blue. *Lane A* represents CP-MVP vaults before crosslinking experiment; *lanes B* and *C* represent CP-MVP vaults after crosslinking before and after centrifugation, respectively, to remove excess crosslinkers; *lane D* represents CP-MVP vaults treated with 25 mM DTT for 1 h after crosslinking to cleave disulfide bond. (b) TEM image of DSP-crosslinked CP-MVP vaults at pH 6.5. (c) TEM image of DSP-crosslinked CP-MVP vaults treated at pH 3.4 for 1 h. Some aggregation is observed as shown by the arrowheads (▶). The inset is an enlarged image of two DSP-crosslinked CP-MVP vaults indicating the cap and barrel structure. (d) TEM image of DSP-crosslinked CP-MVP vaults at pH 6.5 after reduction by DTT. (e) TEM image of DSP-crosslinked CP-MVP vaults at pH 3.4 after reduction by DTT. Half vault structure is resumed as showed by circles.

Table 1

Molecular structures of three sulfhydryl-reactive crosslinkers.

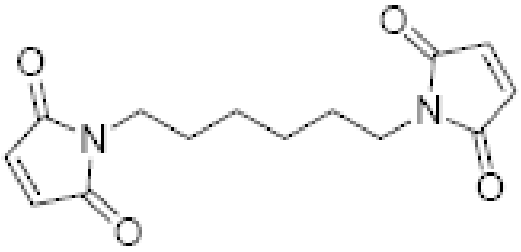
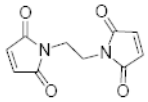
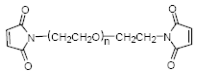
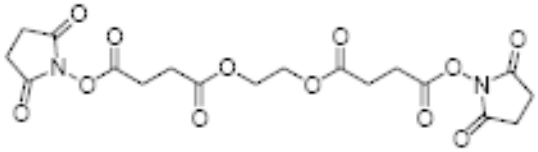
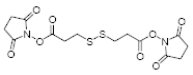
sulfhydryl-reactive crosslinker	structure	length of spacer arm
bis-maleimidohexane (BMH)		16.1 Å
bis-maleimidoethane (BMOE)		8.0 Å
MAL-PEG-MAL (n = 78)		~ 34 nm

Table 2

Molecular structures of two amine-reactive crosslinkers.

amine-reactive crosslinker	structure	length of spacer arm
ethylene glycol bis[succinimidyl-succinate] (EGS)		16.1 Å
dithiobis [succinimidyl-propionate] (DSP)		12.0 Å