The adsorption of biological peptides and proteins at the oil/water interface. A potentially important but largely unexplored field

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Abstract This review focuses on some new techniques to study the behavior of peptides and proteins bound to oil droplets. We will show how model peptides e.g., amphipathic α helices (AαH) and amphipathic β strand (AβS) and some apolipoproteins adsorb to triacylglycerol (TAG) droplets and how they behave once adsorbed to the interface. While most of the studies described involve peptides and proteins at an oil/water interface, studies can also be carried out when the surface has been partially covered with phospholipids. This work is important because it examines biophysical changes that take place at lipid droplet interfaces and how this may relate to the metabolism of lipoproteins and lipid droplets.—Small, D. M., L. Wang, and M. A. Mitsche. **The adsorption of biological peptides and proteins at the oil/water interface. A potentially important but largely unexplored field.** *J. Lipid Res.* 2009. **50:** S329–S334.

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This review centers on some new methods which can be used to study the physical properties of proteins which bind to lipid droplets (1–5). Lipid droplets are found universally among living organisms from primitive prokaryotes to mammals and plants (6, 7). Lipid droplets carry a variety of names including lipid bodies, fat droplets, adiposomes, oil bodies (plants), milk fat globules, lipoproteins, etc. All of these lipid droplets are emulsions consisting of a core of one or more very hydrophobic lipids including triacylglycerol (TAG), wax esters (WE), cholesterol esters (CE), retinal esters,and poly(hydroxyalkanoates) (6).

TAG, wax esters, CE, and retinal esters are highly hydrophobic molecules possessing only a small polar group, the ester group (8). When an emulsion of these lipids is made in an aqueous phase by shaking or sonication, it rapidly coalesces into a single oil layer separated from the aqueous phase. Surface tension (γ) is the energy it takes to create one new cm^2 of surface (i.e., $\gamma = ergs/cm^2$ or mN/m). When a new surface is formed by agitation, the energy is rapidly retrieved by coalescence as total surface reduces to its minimum.

In all biological organisms where droplets are found, the hydrophobic lipid is found as a core droplet surrounded by emulsifiers that decrease γ allowing the droplet to remain suspended without coalescing. These emulsifiers also often provide a charged or bulky surface layer that resists coalescence when droplets collide. Thus, all lipid droplets are emulsions of a hydrophobic lipid separated from the aqueous phase by a layer or layers of emulsifying agents.

In biological systems the emulsifiers are phospholipids, presumably the continuous phase on the surface, and various adsorbed peptides. Protein free TAG and CE emulsions can be made by agitating them with phospholipids (9–11). However, they are readily broken when centrifuged, which separates a surface emulsifying phase from the core lipid (9–11). Specific proteins added to the interface will greatly stabilize it against coalescence. The peptides bound to emulsions also determine the fate of the emulsions. For instance, they protect seed bodies from desiccation or excess hydration so that sequestered TAG can be stored under a variety of hostile environments (6, 7).

In bacteria, higher animals, and plants, lipid droplets vary greatly in size but are usually round (6), indicating a tendency to minimize the interfacial area and decrease the interfacial free energy. They can abut one another without fusing, indicating that the emulsion is stable and that

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Abbreviations: AαH, Amphipathic α helices; AβS, Amphipathic β strand; ADRP, adipocite differential related protein; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoC, apolipoprotein C; apoE, apolipoprotein E; CE, cholesterol esters; CPAT, constitutive PAT; CSP, consensus sequence peptide; PAT, Perilipins/ADRP/TIP47; TAG, triacylglycerol; WE, wax esters.

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hydrophobic molecules at the core do not come in contact between droplets, which would initiate fusion. In some cases, particularly in bacteria grown on saturated alkanes or saturated alcohols, saturated WE are formed that form rods and plates within the bacteria (6). These are probably crystalline deposits reflecting the high melting temperatures of saturated esters of long chain fatty acids and alcohols (8).

PROTEINS ASSOCIATED WITH LIPID DROPLETS

The great interest in obesity and its control has stimulated new research on the metabolism of fat, its storage, its hydrolysis and usage, and more recently, on the nature of lipid droplets in cells (6, 7, 12–15). Certain families of proteins have been described that associate either constitutively or transiently with lipid droplets. A group of proteins called the PAT proteins, consisting of perilipins, adipocyte differential related protein (ADRP), and TIP47, are key peptides in fat droplet assembly and metabolism (12, 16).

PAT proteins form two general classes: *1)* constitutive (CPAT), consisting mainly of perilipin A and ADRP, and *2)* exchangeable PAT, consisting of several other analogous proteins (14). The CPATs remain bound to the lipid droplet from synthesis to lipolysis, while exchangeable PATs may come off as enzymes that are attracted to the surface to hydrolyze the TAG. The CPATs contain lipid binding domains within the central part of the peptide (17).

Fat bodies of plant seeds are very stable (7). They depend on the presence of an interesting peptide called oleosin. Oleosin consists of N- and C-terminal charged amphipathic α helices (AαH) domains that lie on the surface of the droplet and a central ∼60aa hydrophobic β sheet that penetrates into the core lipid. This β sheet interacts with adjacent β sheet central domains to produce an almost rigid surface (18, 19). Interdigitated between and probably under the terminal N- and C-umbrella of the oleosin molecule are phospholipids rendered resistant to hydrolysis (7). Only when appropriate circumstances for germination occur is there hydrolysis of some of the peptides at the interface and subsequent utilization of lipids for new cell growth.

Milk fat globules are secreted with a membrane around the emulsion droplet and this membrane contains a variety of proteins that protect and stabilize the milk fat globules. TAG rich chylomicrons and very low density lipoproteins (VLDL) are other forms of emulsion particles that are generated in the intestines and liver, respectively (20, 21). Budding of these nascent particles occur from the endoplasmic reticulum into the lumen (22) and after some modifications they are secreted into plasma (21).

Once chylomicrons and VLDL enter the plasma they bind apolipoprotein Cs (apoCs) and apolipoprotein E (apoE), and TAG and phospholipids are hydrolyzed by lipases in capillaries delivering fatty acid to the fat, heart, and muscle (20, 21). As VLDL is metabolized it loses most of its TAG, phospholipids and exchangeable apolipoproteins and it becomes much smaller and enriched in CE. This end product is called low density lipoprotein (LDL) and the only protein it contains is apolipoprotein

B (apoB) (20, 21). LDL does not have adequate phospholipids and free cholesterol on the surface to form a stable interface and, therefore, the apoB must bind to the core to stabilize the particle (23). It has been estimated that apoB covers 20 to 45% of the CE core surface (23). While it is certain that apoB interacts with the core lipid in LDL, it has not been specifically established in VLDL or chylomicrons that apoB interacts directly with the core but we suggest that some domains do interact with core TAG (24).

BIOSYNTHESIS OF LIPID DROPLETS

There has been a recent surge of interest in the role proteins play in lipid droplet metabolism. In a provocative paper from Robert Farese's laboratory, Guo et. al. (25) studied cellular lipid droplet formation in *Drosophila* Schneider 2 cells, a system that develops lipid droplets over 24 h in the presence of 1 mM oleate. When the oleate is removed, the cells utilize the lipid. Guo et al. (25) imaged the size and number of droplets in five separate stages during TAG storage. In the first stage, a few small TAG droplets were formed. In the second stage, a larger number of dispersed small droplets were formed. In the third, more droplets were formed but now some of the droplets were larger, suggesting either fusion or continued synthesis on droplets. In the fourth stage, the droplets appeared to clump and appear larger. And in the fifth, only a few very large droplets were present, apparently the result of droplet fusion.

These workers studied the utilization of the TAG when oleate was omitted and showed that the droplets get much smaller and perhaps undergo fission. They report a genome wide RNAi screen of the cells during the five stages of lipid droplet formation and during lipid utilization and report that an astounding 1.5% of all genes appear to have some function in the droplet formation and regulation. Genes encoding lipid biosynthetic-proteins were involved in droplet size and a subset of Arf-COP-I transport proteins also regulated morphology and utilization. A variety of other proteins appeared to have important effects and to interact with the lipid droplets. This study points to a vast number of transient or more constitutive interactions of various proteins with lipid droplets during their formation and catabolism.

STUDYING THE BINDING OF PEPTIDES TO LIPID DROPLETS

Several years ago we began to develop methods to look at the adsorption of peptides, largely derived from human apolipoproteins, to different interfaces including air/water, dodecane/water (2, 4), and TAG/water (4) using a modified drop tensiometer (26). Others devised similar apparatus to study adsorption and desorption of surfactants including a few proteins at the air/water and hexadecane/water interfaces (3, 27, 28). The protocols that we described below for studying interfacial behavior of proteins

derived primarily from our experience using the apparatus described in reference 26. The apparatus (1, 26) consists of a thermostated stirred cuvette and a computer automated syringe that can deliver a bubble of gas or a drop of oil into the aqueous phase in the cuvette. Drop size can be varied between 2 and 30 μl. We added a delivery pipette for adding solution (buffer with or without added surfactant) and a suction pipette that constantly removes the excess solution from the surface while solution is being added to keep the volume in the cuvette constant. This deliver-removal system allows for the complete exchange of solution in the cuvette and can be used to either deplete the cuvette of surfactant (exchange in buffer) or to exchange in surfactant or peptide. The drop shape is continuously projected
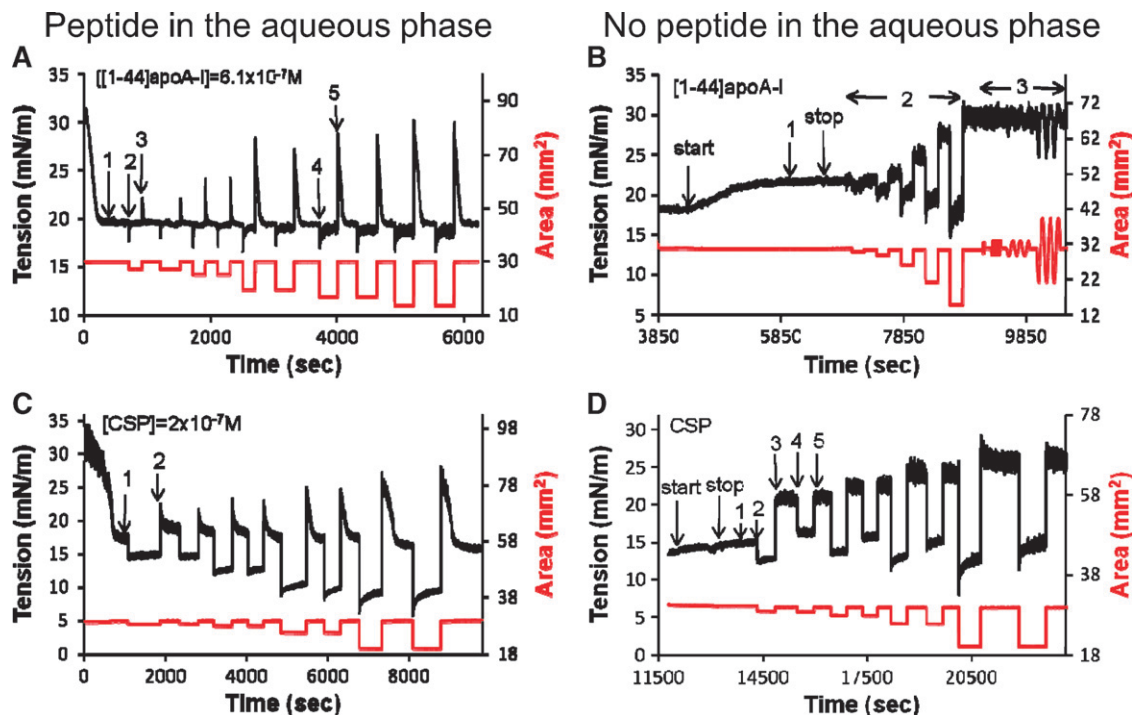
Fig. 1. Amphipathic α helical (AαH) Peptide adsorption to the triolein/water (TO/W) interface and the response to stress compression and reexpansion with peptide in the aqueous phase (left) and after peptide has been washed out (right). Examples of behavior of three different peptides are shown in A and C (peptide at concentration noted). The peptide solution is removed between "start" to "stop" (B and D). A: [1-44] apoA-I, the N-terminal G* type (29) helix of apoA-I (32). A droplet of TO (area = 30 mm²) is formed in the peptide solution. Surface tension γ falls rapidly from 32 mN/m reaching equilibrium (γ_{eq}) at point 1. At point 2 area is decreased by 10%, producing a small decrease in γ that rapidly returns to γ_{eq}. Thus, on sensing the increased π, the peptide ejects from the surface into the bulk. Point 3, the area is reexpanded to 30 mm², immediately producing a spike in γ, but as the peptide rapidly readsorbs from the bulk γ_{eq} is reestablished. In points 4 and 5, larger compression and reexpansion show similar behavior. However, the larger compression (point 4) pushes more peptide off the surface and reexpansion at point 5 produces more free area resulting in the higher γ immediately after the reexpansion. Peptide then readsorbs to reestablish γ_{eq}. B: [1-44]ApoA-I after peptide is exchanged out of solution. Starting at γ_{eq} (~19 mN/m) the peptide is exchanged out between "start" and "stop." During exchange γ_{eq} rises quickly and establish a new γ_{eq} at ~22 mN/m (point 1). Region 2 shows compression and reexpansion after peptide removal. Each compression produces a fall in γ but on reexpansion γ is increased above γ_{eq}. The larger the compression the greater the postexpansion γ. After expansion no change occurs in γ because there is virtually no peptide in the aqueous phase to readsorb (compare with points 3, 5, Fig. 1A). The final γ approaches 32 mN/m, like pure TO. However, large area oscillations (region 3, far right) causes γ to oscillate between ~32 and 25 mN/m, indicating that even when γ approaches 32 mN/m after the last compression some peptide remains bound. To push this peptide off γ must be decreased below γ_{eq} of 22 mN/m (i.e., π > 10 mN/m). C: CSP, a consensus 44aa amphipathic α helix of A type (29). It consists of two identical 20 a.a. helices linked by a proline turn (2, 34). The peptide adsorbs and reaches equilibrium at γ of ~17 mN/m. A small compression lowers γ_{eq} to ~14 mN/m (point 1), which slowly moves back toward equilibrium. On reexpansion (point 2) γ immediately increases, above γ_{eq} to about 23 mN/m. This shows that molecules that were displaced by compression (point 1), readsorb after reexpansion moving γ back to γ_{eq}. Larger compressions (right) show larger changes of similar nature. In these experiments, the post compression increase in γ could be due to peptide molecules leaving the interface or to a conformational change in the peptide at the interface. At the smallest compression we think CSP stays bound but undergoes a conformational change that is rapidly reversible on reexpansion. At all the larger compressions (beyond point 1), CSP is ejected into the aqueous phase, joins bulk peptide, and must diffuse back to the surface to bind. D: CSP is exchanged out between "stop" and "start." Point 1, no significant change in γ (~15 mN/m) occurs during or after exchange (compare with Fig. 1B above). Four sets of two identical compressions were made. Point 2, a small compression decreases γ to ~12 mN/m and on reexpansion (point 3) γ rises to ~21 mN/m. This shows that compression pushed some peptide off. A second identical compression (point 4) reduces a γ to ~16 mN/m, i.e., ≅ γ_{eq}. On reexpansion (point 5) γ returns to ~21 mN/m. Thus, the second compression does not push more peptide off the surface but simply compresses it. Larger compression (right side) show that the first compression dislodges peptide but the second does not. Thus, if compression does not push γ below γ_{eq} no peptide is desorbed.

on a charged coupled device (CCD) camera and the image analyzed by computer to give an almost continuous measure of drop volume, area, and γ. By changing volume, area can be decreased or increased causing compression or expansion of the surface. The area can be sinusoidally oscillated to generate γ/area curves from which the dilatational elastic modulus ε and its real (purely elastic) ε' and imaginary (viscous) ε'' parts can be estimated (1). Our studies show that adsorption of the peptides results in lowering of γ thus decreasing the surface free energy of the droplet. The data indicate that some peptides can be pushed off the surface by increasing the interfacial pressure (π), while other peptides can withstand great π without being ejected from the surface.

Amphipathic α helices (Fig. 1)

The (AαH) motif is the dominant secondary structure of all exchangeable apolipoproteins (29). It also makes up about 50% of apoB domains (30). Many other lipid binding proteins also have AαH (31). We have identified two classes of AαH that differ primarily in their ability to lower γ and to exchange off the surface. The first group are partially exchangeable AαH. Examples of these types of peptides include the N- and C- terminals of apolipoprotein A-I (apoA-I) ([1-44]apoA-I, [198-243]apoA-I) (32) and the small apolipoprotein apoC-1. They bind rapidly to the triolein/water (TO/W) interface, but all are pushed off by any surface pressure (π) greater than their equilibrium π ($\pi_{eq} = 32$-γ_{eq}) (**Fig. 1A**). Further, as the peptides are exchanged out of the aqueous phase the γ rises during the exchange from γ_{eq} to a new higher γ_{eq} plateau (Fig. 1B). This suggests that there are two bound forms that do not appear exchangeable on the interface; one that stays permanently bound and gives rise to the γ after the washout (new γ_{eq}) and a second more weakly bound one that is able to diffuse off and be removed during the exchange process. The bound form is very stable but when surface area (A) decreases, π increases (γ decreases), which immediately pushes the peptide off the surface into the aqueous phase (point 2, Fig. 1B).

A second type of adsorption is illustrated by CSP, a consensus 44aa peptide of helices of apoA-I, apoA-II and apoE. This peptide forms an antiparallel 2 helix bundle around a central proline initiated turn. This peptide binds rapidly but when the droplet is compressed, peptide desorption is much slower (Fig. 1C) and γ does not return to γ_{eq} even after several minutes. Some of the peptide is ejected, as shown by the peak in γ above γ_{eq} when the area is returned to normal (Fig. 1C, point 2). When CSP is exchanged out of the aqueous phase (Fig. 1D) no change occurs in the γ_{eq}, indicating that the peptide that was bound in the presence of peptide does not come off either during exchange or afterwards. Thus, these peptides are characterized by extremely slow off rate.

Both the partially exchangeable and non-exchangeable AαH peptides can be pushed off the surface as shown by Figures 1B and 1D. After removal of aqueous peptide, each compression pushes some peptide into the water and when the A is raised back the γ is much higher, indicating the peptide has left the surface. With each larger compression more peptide is pushed off the surface. However, even when the γ approaches that of pure triolein (Fig. 1B), there is still peptide on the surface because the γ falls when compression occurs. Even if a second exchange is carried out at this point (data not shown) peptide remains on the surface. We speculate that if the hydrophobic part of AαH was somewhat larger then the peptide might be more difficult to push off or be irreversibly bound.

Amphipathic β Strands (Fig. 2)

Amphipathic β strands (AβS) show alternating hydrophobic and hydrophilic residues and are an ideal structure to bond to a hydrocarbon interface (4). They strongly adsorb to TAG/water, dodecane/water, and air/water interfaces, decreasing γ more than the AαH (2, 4) (**Fig. 2A**). There is no evidence that these peptides are pushed off
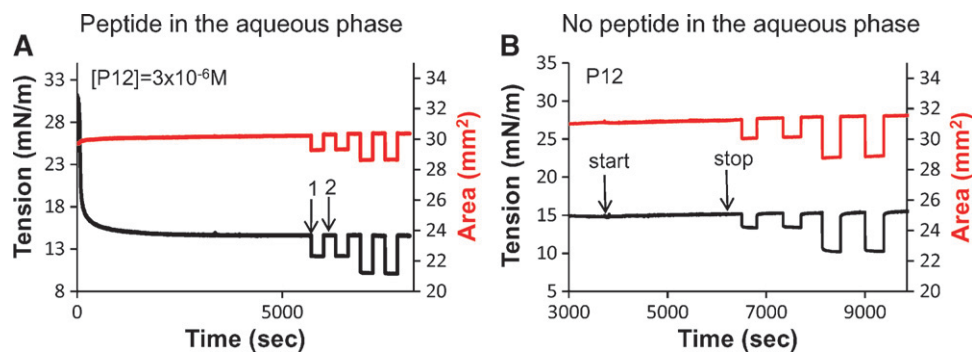
Fig. 2. Amphipathic β peptides (AβS) A: P12, 12aa β strand (acetyl-LSLSLNADLRLK-amide) consensus sequence from TAG recruiting domains of apoB (4). At γ_{eq}, ($\sim$14 mN/m)γ a moderate compression (point 1) decreases γ but there is no change after the compression. When the compression is released (point 2) returns to γ_{eq}. A larger compression (right) decreases the γ further, but γ on reexpansion returns to γ_{eq}. Such behavior indicates that the peptide does not leave the interface or have a major conformation change when compressed. B: P12 after the peptide is removed. During exchange no peptide desorbs from the interface and compression reexpansion experiments are almost identical to Fig. 1E. P12 is irreversibly bound and behaves as an elastic solid.

the surface either before the exchange (4) or after the peptide has been exchanged out (see Fig. 2B). The AβSs are fully elastic (4) both before and after the peptide is washed out (data not shown). These peptides are what probably anchor apoB to the interface of LDL and TAG-rich lipoproteins (24). Although the lipid binding regions of olesins, Hepatitis-C virus peptides (33), perilipin A, and some caveolins have not been studied, we would predict these peptides would irreversibly bind to the TAG/water interface.

Larger complex peptides and native proteins

Complex interfacial behavior is often shown by larger full length apolipoproteins (24, 34) and some of the multidomain fragments of apoB (35). ApoA-I shows a π sensitive differential desorption phenomena (34). At relatively low π, between ~15 and 20 mN/m, some α helical segments are ejected from the surface on compression. Because part of apoA-I remains bound, these ejected domains snap back rapidly onto the surface on reexpansion (34). When π is above 20 mN/m, apoA-I is totally ejected from the interface and passes into the aqueous phase (34). When the free apoA-I is exchanged out of the aqueous phase, it remains bound (i.e., it has a very slow off rate). However, when adequate π is applied, apoA-I can be pushed off the surface into the aqueous phase, like CSP (Fig. 1D), but at higher π ($\pi > 20$ mN/m).

We have shown that full length apoB adsorbs to TAG droplets and once adsorbed cannot be pushed off the surface (24). However, it undergoes π related conformational changes at the interface presumably related to some motifs (probably AαH) being disengaged at high π and rebinding rapidly at low π. These changes are reversible, but as a result, apoB has a small viscous component related to this surface conformational change even though it is mainly elastic (24). This would allow it to be an excellent buffer to changing π conditions that occur during metabolism of TAG-rich lipoproteins.

The region between apoB37 and apoB41 of apoB was originally implicated to be a TAG recruiting domain (24). It consists mainly of AβS and we have now shown that it behaves in a very similar way to AβS shown in Fig. 2 when adsorbed to TAG drops (unpublished observations). On the other hand, a peptide from the N-terminal, apoB6.4-17, which contains both a large α helical region and a globular α helical/β sheet domain, behaves in a very different fashion (35). About 40% of the protein does not bind to the TAG interface, but seems to form a suspended globule. The ~60% bound part can undergo a reversible pressure-sensitive conformational change (35).

It is important to stress that peptides coming from the apolipoprotein family and presumably those coming from other constitutive peptides bound to lipid droplets are specifically evolved to bind lipid and are different from many water soluble globular proteins. Surface chemists for many years have studied the interfacial behavior of groups of available peptides (albumins, betalactaglobulins, β casein, lysozyme, etc.) at the air/water interface (1, 27, 28). These peptides all bind irreversibly to the air/water interface. Several appear to undergo at least two stages during adsorption at the hexadecane/water interface, the first being binding of the intact tertiary structure and the second, the slow unfolding of the tertiary structure at the interface to form a network of denatured peptide irreversibly tangled at the interface (3).

We have looked at both lysozyme and apoprotinin (BTIP), which are small water soluble well-folded peptides with stabilizing disulfide bonds. At the TAG/water surface, both adsorb slowly to the interface at 1 μM with the γ gradually falling over many hours, unlike the rapid adsorption of peptides discussed earlier (Figs. 1 and 2). When the peptide is removed, very little desorbs and when the droplets are compressed, the peptides cannot be pushed off the surface (unpublished observations). Thus, once the peptides adsorb and then unfold, positioning their hydrophobic residues in the TAG interface, an irreversible binding takes place and the peptides act as an elastic layer at the interface. Unlike the AαH of plasma lipoproteins, which can be pushed off the hydrophobic surface and retain their secondary and probably tertiary structure in solution, ordinary water soluble globular peptides once adsorbed are dead.▣

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