RESEARCH COMMUNICATION

S–S bonds are not required for the sonochemical formation of proteinaceous microspheres: the case of streptavidin

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Proteinaceous microspheres can be prepared using the sonochemical method. However, it is known that these proteins should possess at least one cysteine residue in order to obtain stable microspheres using this method. In the present study, we have produced streptavidin microspheres, using the sonochemical

method, from streptavidin, which does not have any cysteine residues.

Key words: cross-linking, sonochemistry, streptavidin microspheres, ultrasound.

INTRODUCTION

Microspheres, microscopic vesicles, have wide ranging applications, including micro-encapsulation of dyes, flavours and fragrances, drug-delivery systems and magnetic resonance contrast (and imaging) agents [1–8]. Previous work by Suslick and co-workers has shown that micron-sized gas- or liquid-filled proteinaceous microspheres can be produced from BSA with high-intensity ultrasound [9–11] and also from human serum albumin [12]. Gas-filled Hb microspheres have also been produced using the sonochemical method [13]. These microspheres are formed by chemically cross-linking cysteine residues of the protein with HO_2^- (superoxide radical) around a micron-sized gas bubble or non-aqueous droplet. Following these results [13], we have succeeded in producing avidin microspheres using the sonochemical method (S. Avivi and A. Gedanken, unpublished work). Furthermore, in the present study we have tried to extend this method to streptavidin, a protein very similar to avidin, and synthesize its microspheres. It is well known that streptavidin, a 60 kDa protein produced by *Streptomyces avidinii*, forms a very strong and specific non-covalent complex with the water-soluble vitamin biotin [14,15]. The protein comprises four identical subunits, each with an approximate molecular mass of 15 kDa. It binds 4 mol of biotin/mol of protein and does not contain any carbohydrate. Avidin is usually isolated from chicken egg whites, and shares some common characteristics with streptavidin, such as molecular mass, subunit composition and the capacity to bind biotin, forming a complex of very high affinity (K_d is approx. 10 fM) [16,17]. Both proteins belong to the protein class containing β -barrel structures and have an unusually high content of threonine and tryptophan. Whereas avidin bears a single tyrosine, the content of tyrosine residues in streptavidin is high (six per subunit). Avidin is a glycoprotein containing a disulphide bridge and two methionine residues, whereas streptavidin is non-glycosylated and devoid of any sulphur residues. As mentioned above, this sulphur residue is necessary for the formation of the microsphere when using the sonochemical method. In the present study, we have reported the extension of the sonochemical

method to a non-sulphur-containing protein, streptavidin. These microspheres are stable for many hours at room temperature (20–25 °C) and for at least 1 month at 4 °C. A mechanism different from that of the formation of S–S bonds is proposed to account for these results.

EXPERIMENTAL

Streptavidin (Sigma) was used without any further purification for the synthesis of proteinaceous microspheres, as described previously [18]. Decanlin was layered over a 5% (w/v) protein solution. The bottom of the high-intensity ultrasonic horn was positioned at the aqueous–organic interface. The mixture was irradiated for 3 min under open air conditions (101.325 kPa), employing an acoustic power of approx. 150 W/cm² (20 kHz) with an initial temperature of 20 °C in the reaction cell. No microspheres were obtained when the pH was maintained at 7. However, when the pH was lowered to 6.0 by adding conc. HCl, microspheres were formed. It is very important to note that adding conc. acid did not create any local denaturation. To demonstrate this, we also conducted the reaction under mild conditions using a buffer (pH 6) and obtained the same microspheres. The sonication of streptavidin was also conducted under 151.988 kPa of argon, keeping all the other conditions as in the reaction under air.

After the synthesis, the product was separated from the unreacted protein, and was then washed several times with sufficient volumes of water. The morphology of these materials was determined using scanning electron microscopy (SEM) (scanning electron microscope, JSM-840; JEOL, Peabody, MA, U.S.A.). The sample was prepared for SEM by secondary cross-linking with glutaraldehyde and by coating with gold. The microsphere size distribution in the solution was measured using a DLS instrument (GALAI-CIS 1; Migdal Haemek, Israel). This is a computerized inspection system for particle size analysis, which operates in the range 0.5–20 μm .

Abbreviation used: SEM, scanning electron microscopy.

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RESULTS AND DISCUSSION

Figure 1 is a scanning electron micrograph of the streptavidin microspheres, which has some cracks on the surface. The morphology of the microspheres made of streptavidin is very similar to that obtained in previous studies [9–13]. The size distribution of the microspheres is shown in Figure 2. A broad size distribution is obtained for the product with an average diameter of approx. 5 μm .

As stated above, we started to obtain microspheres only at pH lower than 6.0, whereas using the same method at neutral pH did not yield any product. Thus an acidic medium is crucial for the preparation of the streptavidin microspheres. The role of the acidic medium in sonochemical mechanism is explained as follows. Ultrasonic irradiation of liquids is well known to produce both emulsification [19] and cavitation [20–22]. Emulsification alone, however, is insufficient to form long-lived microspheres. It was found that the microspheres are held together by protein cross-linking through disulphide linkages from cysteine oxidation [9–13]. However, it is obvious that this is not the mechanism for the formation of streptavidin microspheres. Suslick and co-workers [9–13] have interpreted the formation of the S–S bonds as being due to the oxidation of the SH groups of the cysteine residues by the superoxide radicals formed in the sonication of water under air. In the present study, unlike those with avidin (S. Avivi and A. Gedanken, unpublished work), BSA [9–11], human serum albumin [12] and Hb [13], microspheres of streptavidin were formed even when the solution was sonicated under argon. This proves that the mechanism for the formation of microspheres in streptavidin is different from that operating in the formation of the other proteins due to the absence of SH functional groups.

According to our explanation, hydrophobic or thermal denaturation of the protein after the initial ultrasonic emulsification assists in microsphere formation. According to Lins and Brasseur [23], the hydrophobic effect drives the molecules towards a more condensed structure by decreasing the unfavourable contacts between the hydrophobic residues and water molecules. A minor contribution is provided by the lowering of the pH, which helps to neutralize the basic carboxyl edges, thus creating a more favourable hydrophobic environment. To verify this hypothesis, we sonicated a poly(glutamic acid) protein (Sigma), which has only carboxy groups on the side chains. The con-

Figure 1 Scanning electron micrograph of streptavidin microspheres

The microspheres were prepared for SEM by secondary cross-linking with glutaraldehyde and by coating with gold.

Figure 2 Particle distribution of an aqueous suspension of streptavidin microspheres determined with a Coulter particle analyser

centration of the solution was 5 % (w/v) protein, with all the other sonochemical parameters remaining unchanged. Microspheres of the poly(glutamic acid) were formed only at a pH lower than 4.5. This pH is lower than the pK_a of the side chain of this polymer (the pK_a of the side chain carboxy groups of aspartic acid and glutamic acid residues is 4–4.8). Thus we can conclude that hydrophobic interactions, which become more dominant in acidic medium, are responsible for the production of the microspheres in poly(glutamic acid), as well as in streptavidin. Those hydrophobic interactions are strong enough to produce microspheres, which are stable for at least 1 month.

We also checked the ability of streptavidin microspheres to bind biotin compared with commercially available streptavidin. It was found that streptavidin microspheres bound approx. 50 % of the amount of biotin compared with the amount of biotin that is bound to the native protein. We have found that the biological activity of proteinaceous microspheres is reduced by 30–50 %, as compared with the native protein (S. Avivi and A. Gedanken, unpublished work).

In conclusion, the fact that we can form streptavidin microspheres enlarges the limited number of proteins that can undergo microspherization in an ultrasonic field, from cysteine-containing proteins to a much larger variety of proteins.

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