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ORIGINAL CONTRIBUTION

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Spherical hydrophilic microparticles obtained by the radical copolymerisation of functionalised bovine serum albumin

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Abstract Unsaturated groups were introduced onto proteins in order to produce macromers that are able to undergo radical polymerisation. The initial protein used was bovine serum albumin (BSA) because it is biodegradable, biocompatible and easily available. Methacrylic groups were introduced onto BSA by reaction with methacrylic anhydride at controlled pH and temperature. The experimental conditions allowed the protein to be kept water-soluble. This water-solubility of the derivatised protein was essential when realising spherical polymeric microparticles via reverse phase suspension copolymerisation with *N*,*N*-dimethyacrylamide (DMAA). During the derivatisation, the insertion of the polymerisable groups affects only the sterically-available chemical functions of the native protein. Therefore, chain growth during the copolymerisation process involves only these groups, achieving a polymeric network around a structurally unmodified protein. The polymeric systems show high water affinity, ascribable to the hydrophilic properties of BSA. We have demonstrated that the achievement of the spherical form during the polymerisation depends on two factors: the degree of derivatisation of BSA, and the weight/weight (w/w) ratio of the protein to the comonomer. The beads obtained were characterised by Fourier transform IR spectrophotometry, particle size distribution analysis, and scanning electron microscopy (SEM). The samples investigated showed a remarkable affinity for water and a high swelling capacity. These properties depend upon the degree of derivatisation of BSA and on the percentage of DMAA in the copolymerisation mixture. In this paper we describe the starting materials and the experimental conditions used to prepare protein hydrogels by radical copolymerisation, which are intended for use in pharmaceutical and biomedical applications.

Keywords Acryloylated bovine serum albumin · Reverse-phase suspension polymerisation · Spherical microparticles · Hydrogels

Introduction

In recent years the strategy of utilising microspheres as carrier systems for drug delivery has gained increasing interest [1, 2, 3, 4, 5, 6]. Microspheres can be used in the treatment of many diseases that require controlled release of the drug into plasma, cells or organs. Considerable interest has been shown in the use of protein microspheres as starting materials for active drug targeting, as well as for producing a sustained and controlled rate of drug

release [7, 8, 9]. The albumin microspheres extensively studied in previous works are suitable for drug delivery since they are biodegradable, biocompatible and relatively easy to prepare over a wide range of particle sizes. They are reported to be relatively non-toxic and non-immunogenic [10, 11, 12, 13, 14, 15, 16]. Crosslinking of albumin can be achieved by thermal denaturation, either by direct reaction between functional groups (usually carboxyl and amino groups) in polypeptide side chains, or via chemical crosslinking agents such as glutaraldheyde, formaldehyde and 2,3-butanedione [17, 18, 19]. Albumin microspheres were prepared by stirring an aqueous solution of albumin and adding a crosslinking agent to the organic phase. The resultant water-in-oil emulsion was heated (115 ± 5 °C) until the microspheres were essentially dehydrated. After drying, the microspheres are filtered and washed. The diameters of the microspheres depend on the stirring speed, while the rate of biodegradability is tailored by the heating. In chemical crosslinking, the amount of crosslinking agent determines the structural strength and textural tightness.

All previous techniques used hydrophobic materials that released the drugs through an erosion mechanism.

To avoid the physical denaturation of the protein during the stabilisation process, previous works described a technique, which, by maintaining the temperature below 37 °C (preferably 4 °C) and the pH at 5.5–8.5 throughout the process, produces hydrophilic blend materials [20, 21] where human serum albumin (HSA) is dissolved in a solution of polymethylmethacrylate and stabilised by glutaraldehyde.

In the present study, we report the chemical modification of BSA by introducing unsaturated groups onto it, and its subsequent employment as a macromer used for preparing spherical microparticles by a radical copolymerisation mechanism. During the derivatisation process, the protein tertiary structure is largely maintained, so that the derivatised BSA (BSA_f) is water-soluble. Radical copolymerisation of BSA_f with *N*,*N*-dimethyacrylamide (DMAA) produced a polymeric network built around a structurally unmodified protein. This material shows a high affinity for water. The hydrophilicity of each bead of this network allows us to incorporate high concentrations of water-soluble drugs into the spheres after synthesis.

The technique used in this work has various advantages. The systems obtained by this radical mechanism are characterised by proteins covalently bonded together into a polymeric network which are more stable than systems linked by weak interactions. The radical polymerisation permits the realisation of versatile materials. Varying the nature and amount of comonomer is possible in order to be able to realise protein hydrogels with different properties that can be used in the biomedical and pharmaceutical fields.

In order to synthesise particles with various structures and crosslinking densities, hydrogels formed from BSA_f and DMAA in different ratios (w/w) were prepared. BSA was partially modified by reaction with methacrylic anhydride (MA), under controlled pH and temperature, obtaining a water-soluble material. In particular, a series of modified BSA samples were obtained by varying MA concentration and reaction time. The degree of acylation (DD%) was determined spectrophotometrically [22, 23]. By varying DD% and the w/w ratio between BSA_f and comonomer (DMAA) in the feed polymerisation we have obtained materials with different morphological properties. Our interest focused upon producing spherical geometry, useful for pharmaceutical applications because it eliminates the anisotropic swelling behaviour normally associated with other geometries (planar or cylindrical) [24].

The beads obtained were characterised by Fourier transform IR spectrophotometry, particle size distribution analysis, scanning electronic microscopy, and swelling behaviour.

Experimental

BSA fraction V (MW 68.000; pH 7.0 ± 0.2; grade≥98%) was from Roche Diagnostics GmbH. DMAA, *n*-hexane and carbon tetrachloride, purchased from Aldrich Chemical Co., were purified by standard procedures. MA, 2,4,6-trinitrobenzensulphonic acid (TNBS), sorbitan trioleate (Span 85), polyoxyethylene sorbitan trioleate (Tween 85), *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TMEDA) and ammonium persulfate were bought from Fluka Chemical Co.

The dialysis tubes used were 6–27/32″, Medicell International Ltd. The "Freezing-drying apparatus" was from Micro Modulyo, Edwards. Ultraviolet spectra were recorded with a U-2000 Hitachi spectrophotometer using 1 cm quartz cells. FT-IR spectra were recorded as pellets in KBr in the range 4000–400 cm^{-1} using a Perkin-Elmer PARAGON 1000 PC spectrophotometer. The resolution was 1 cm^{-1}. The number of scans was 100. Particle size distribution was carried out using an image processing and analysis system: Leica DMRB equipped with a Leica Wild 3D stereomicroscope. This image processor calculates the particle area and converts it to an equivalent circle diameter. The scanning electron microscopy (SEM) photographs were obtained with a Jeol JSMT 300 A; the surface of the samples was made conductive by the deposition of a layer of gold on the samples in a vacuum chamber.

Determination of BSA derivatisation and degree of functionalisation

After dissolving BSA in distilled water, the solution obtained was treated with a suitable amount of MA

(Table 1) under controlled pH and temperature (pH 7 and 0 °C) conditions and stirred for 1 h at 0 °C. The aqueous solution obtained was introduced into dialysis tubes and dipped into a glass vessel containing distilled water at 20 °C for 48 h with four changes of water. The resulting solution was frozen and dried with "freezing-drying apparatus" to afford a vaporous solid. The degree of derivatisation was determined according to a procedure reported in the literature [23]. Samples were dissolved in 0.10 M sodium tetraborate (pH 9.3). An excess of aqueous 0.03 M TNBS (25 µl) was added to 1 ml of protein solution contained in a cuvette, agitated to ensure complete mixing, and allowed to stand for 30 min at room temperature. The reagent blank consisted of 25 µl of 0.03 M TNBS in 1 ml of 0.10 M borate. Absorbance was read at 420 nm. Unmodified albumin was used as a control, and the degree of acylation was expressed as the percentage of total available amino groups that can undergo functionalisation.

Table 1 Reaction between BSA and MA

BSA (mg)	MA (mmol/mg)	Initial	DD%
500	0.0810/12.5	A	4
500	0.162/25.0	B	22
500	0.268/41.3	C	33
500	0.536/82.6	D	63
500	1.343/207.1	E	100

All reactions were carried out in H_2O (3 ml) at pH = 6.5–7.5 (HCO_3^-/CO_3^{2-} buffer 0.2 M) and $T = 0$ °C

Bead preparation

In a typical experiment, a mixture of *n*-hexane and carbon tetrachloride was placed in a round-bottomed cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 40 °C, then treated, after 30 min of N_2 bubbling, with a solution of modified BSA, comonomer (DMAA) and ammonium persulfate in water. The density of the organic phase was adjusted by the addition of CCl_4 or *n*-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span85 and Tween85, then after 10 min with TMEDA, and stirring was continued for another 60 min. The amounts of all reagents used in these experiments are reported in Table 2. Each matrix obtained was filtered, washed with 50 ml portions of 2-propanol, ethanol, and acetone, and dried overnight under vacuum at 40 °C.

Determination of water regain

Aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5 ml sintered glass filter (diameter 10 mm; porosity, G3), weighed,

Table 2 Homopolymerisations of derivatised BSA and copolymerisations of derivatised BSA with DMAA

Aqueous dispersed phase		Organic continuous phase	Resin	
BSA (mg/DD%)	DMAA (mmol/mg)	CCl_4/Esano (ml/ml)	mg (conv.%)	Initial
45/4	4.55/451	16/25	–	A_1
270/4	2.71/269	15/23	–	A_2
350/4	2.23/221	15/23	–	A_3
450/4	0.455/45.1	15/23	450 (91)	A_4
500/4	0	16/24	475 (95)	A_5
45/22	4.55/451	15/23	–	B_1
270/22	2.71/269	15/23	387 (72)	B_2
350/22	2.23/221	15/23	445 (78)	B_3
450/22	0.455/45.1	15/23	436 (88)	B_4
500/22	0	15/23	475 (95)	B_5
45/33	4.55/451	16/23	–	C_1
270/33	2.71/269	16/23	527 (98)	C_2
350/33	2.23/221	15/23	553 (97)	C_3
450/33	0.455/45.1	15/23	480 (97)	C_4
500/33	0	16/23	465 (93)	C_5
45/63	4.55/451	15/26	–	D_1
270/63	2.71/269	15/23	432 (80)	D_2
350/63	2.23/221	16/23	513 (90)	D_3
450/63	0.455/45.1	15/23	376 (76)	D_4
500/63	0	16/23	315 (63)	D_5
45/100	4.55/451	16/23	–	E_1
270/100	2.71/269	16/23	511 (95)	E_2
350/100	2.23/221	16/23	560 (98)	E_3
450/100	0.455/45.1	15/23	416 (84)	E_4
500/100	0	16/23	400 (80)	E_5

For all polymerisations, the amount of aqueous phase is 3 ml; initiator system is $(NH)S_2O_8$/TMEDA (100 mg/150 µl); surfactants are Span 85/Tween 85 (120 µl/30 µl)

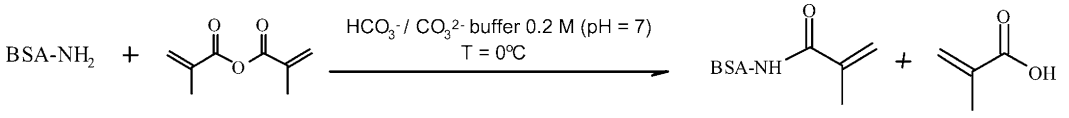
and left to swell by immersing the filter plus support in a beaker containing distilled water. At a predetermined time, the excess water was removed by percolation at atmospheric pressure, by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min and weighed. This operation was repeated at different times (1, 4 and 24 h). The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water regain by the following Eq. 1:

$$WR\ (\%) = \frac{W_s - W_d}{W_s} \times 100 \quad (1)$$

Here W_s and W_d are the masses of the swollen and dried spherical microparticles, respectively (Table 3). Each experiment was carried out in triplicate and the results were in agreement within $\pm 4\%$ standard error.

Results and discussion

Chemical groups susceptible to radical polymerisation were introduced onto BSA by acylating the BSA with MA in water at 0 °C and neutral pH (Scheme 1).



Scheme 1 Derivation reaction of BSA with MA

The nucleophilic chemical groups in BSA that could react with MA are the thyolic groups of cysteine, hydroxilic groups of serine and tyrosine, and amino groups in the side chain of lysine (Table 4). The first are involved in disulfide bridges, except cys-34, the latter are the least nucleophilic, and do not react in mild experimental conditions. It is the sterically accessible amino groups of lysine that react chiefly with acylant agent at

Table 3 Water regain% (WR%) of beads in various media

Heads	Water regain (%)	
	pH = 1	pH = 6.8
B_3	478	480
B_4	184	238
C_3	384	405
C_4	165	200
D_2	567	565
D_3	376	464
D_4	110	140
E_2	292	442
E_3	261	325
E_4	120	287

Table 4 BSA composition

Ala 48	Cys**35**	Asp 41	Glu 58
Phe 40	Gly 17	His 16	Ile 15
Lys**60**	Leu 65	Met 5	Asn 14
Pro 28	Gln 21	Arg 26	Ser**32**
Trh 34	Val 38	Trp 3	Tyr 21

controlled pH and temperature to produce water-soluble BSA_f. If the reaction is carried out without pH and temperature control, denaturation of BSA was observed, and its water solubility is lost.

BSA_f samples with various functionalisation percentages were obtained using different amount of MA. We realised materials with 4%, 22%, 33%, 63% and 100% of the available amino groups acylated. The derivatisation degree was determined by a spectrophotometric method, using TNBS as the chromophore group [23]. In this procedure, TNBS was employed as a reagent for measuring the free amino groups of albumin. The amino content is related to the increase in absorbance at 420 nm that is ascribable to the trinitrophenylsulfonic group bounded to BSA_f, after a relatively short incubation period.

Several samples of BSA_f were crosslinked by radical polymerisation through a reverse-phase suspension polymerisation technique. The reaction was started using TMEDA and ammonium persulfate as initatior.

The polymerisation reaction, owing to steric and geometric constraints, involves only the methacrylic functions of BSA_f which are accessible to the growing chains. The microparticle structure obtained is characterised by a network where the BSA chains are linked by some hydrocarbon bridges. The BSA_f samples were also polymerised in the presence of various amounts of comonomer (DMAA) in order to study the effects of a different chemical structure and degree of crosslinking on the physical properties of the microparticles. It can be supposed that in the copolymerisation reaction the chains obtained consist of DMAA units randomly interrupted by methacrylic BSA_f functions which are sterically and geometrically attainable. Optimisation of the polymerisation method required several attempts. It was observed that hydrophilic/lypophilic balance (HLB) of surfactant and initiator systems is very important. Generally, water-in-oil emulsions are stabilised by surfactants with concentrations of 0.5–1.5% in weight compared to water. In the literature, the best results

were obtained using surfactant mixtures with very different values of HLB [25]. In particular, the volume ratio of surfactants depends on the dispersed phase. In our experiments many tests were carried out, allowing us to determine the correct ratio for Span85 (HLB = 1.8) and Tween85 (HLB = 11). We achieved a system with HLB = 3.0. We also tested $(NH_4)_2S_2O_8$/TMEDA and azobisisobutyrronitrile (AIBN) [24] as initiator systems, but we only observed polymerisation only with the former system. The polymerisations carried out allow us to draw the following conclusions (Table 5):

– The ratio (w/w) between BSA_f and DMAA is important for the realisation of microspheres.
– Homopolymerisation of all of the BSA_f resulted in a mixture of spherical and irregular microparticles (A_5–E_5). An increase in the number of spherical microparticles in the material (although most of the microparticles still had irregular shapes) resulted from increasing the degree of derivatisation of BSA_f.

-Copolymerisation with an excess of DMAA compared to the concentration of BSA_f produces highly swollen (gelled) particles that are usually difficult to recover by filtration (A_1–E_1). In any case, polymerisation of this product gives rise to a poorly crosslinked network.

-Using 4% BSA_f results in material that is not very crosslinked (gelled A_2, A_3; with an excess of protein, irregular microparticles, A_4) were achieved.

-When DD% was 22% and 33%, an excess of protein was needed to form spherical microparticles (B_3, B_4, C_3 C_4). In different cases we observed the formation of gelled or irregular microparticles (B_1, B_2, C_1, C_2).

-An excess of BSA_f (63% or 100% in the polymerisation mixture compared to the amount of DMAA) resulted in spherical microparticles (D_3, D_4, E_3, E_4).

-The same results were obtained using an w/w ratio of DMAA to BSA_f equal to 1 (D_2, E_2).

-Finally, the amount of DMAA required to obtain spherical protein microparticles depends on the degree of functionalisation of BSA_f. When this degree is very high (at least 63%), these spherical protein microparticles can be obtained using large amounts of comonomer, in which case the materials show the chemical and physical properties of the comonomer.

The spherical microparticle samples were characterised by Fourier transform IR spectrophotometry, particle size distribution analysis, and scanning electron microscopy (SEM), and by measuring their water regains in solutions which imitate biological fluids. The water-regain values are shown in Table 3; they were determined in order to assess the affinity of the spherical microparticles for water. It is well known that the swelling of a hydrophilic polymeric network dependings on factors such as the structure of the material, the degree of crosslinking, the hydrophilic/hydrophobic balance, the shape and dimension of the system, and so on. Hydrophilic affinity is proportional to the degree of crosslinking in the samples, which depends on the degree of derivatisation of BSA_f and the amount of DMAA. Samples with a lower DMAA/BSA_f ratio swell more at higher pH probably because modification of the chemical groups in the side chains of the protein influence the pH behaviours of these materials.

In the FT-IR analysis, absorption bands at 2962 and 2868 cm^{-1}, ascribable to the methylic groups of DMAA, appeared in every sample. These bands showed a proportional increase in intensity as the amount of DMAA present in the material increased. Furthermore, a decrease in the intensity of the absorption band at 617 cm^{-1} (a typical band from BSA homopolymers) can be observed as the amount of DMAA increased in the beads. Figure 1 shows IR spectra for samples of E_2 and E_5. Similar results were obtained for all of the samples that we created.

The reverse-phase suspension polymerisation technique represents a simple method for obtaining spherical microparticles whose size can be conveniently varied, according to particular needs, by changing the reaction conditions (speed of stirring, shape and dimension of

Table 5 Characteristics of homo- and co-polymers produced

Ratio DMAA/BSA_r	BSA DD%				
	4%	22%	33%	63%	100%
10	Gel A_1	Gel B_1	Gel C_1	Gel D_1	Gel E_1
1	Gel A_2	IM B_2	IM C_2	**SM** D_2	**SM** E_2
0.6	Gel A_3	**SM** B_3	**SM** C_3	**SM** D_3	**SM** E_3
0.1	IM A_4	**SM** B_4	**SM** C_4	**SM** D_4	**SM** E_4
0	ISM A_5	ISM B_5	ISM C_5	ISM D_5	ISM E_5

SM: spherical microparticles; IM: irregular microparticles; ISM: irregular and spherical microparticles

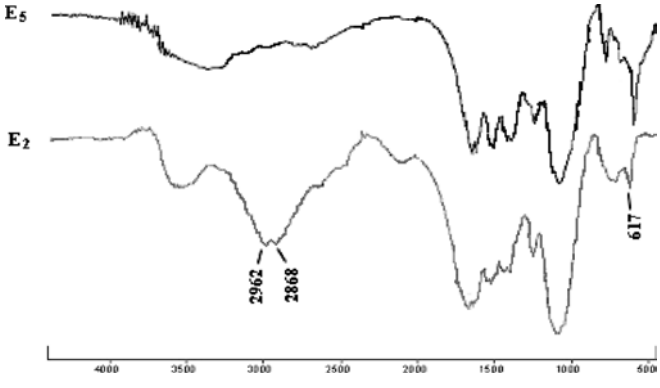
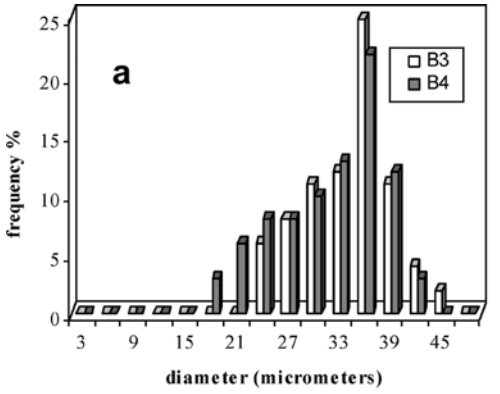
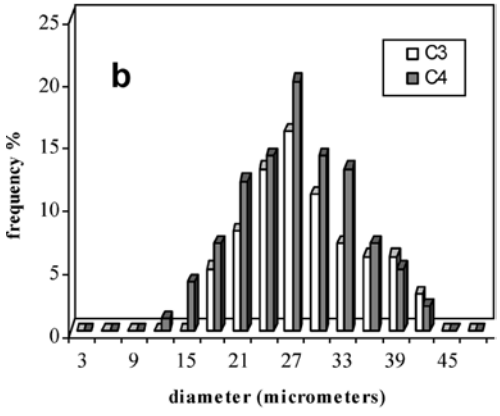
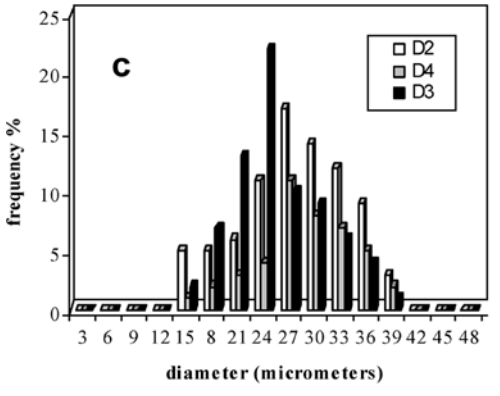


Fig. 1 IR spectra for homopolymer E_5 and copolymer E_2







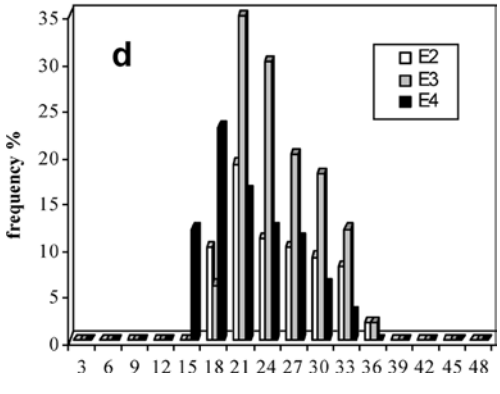


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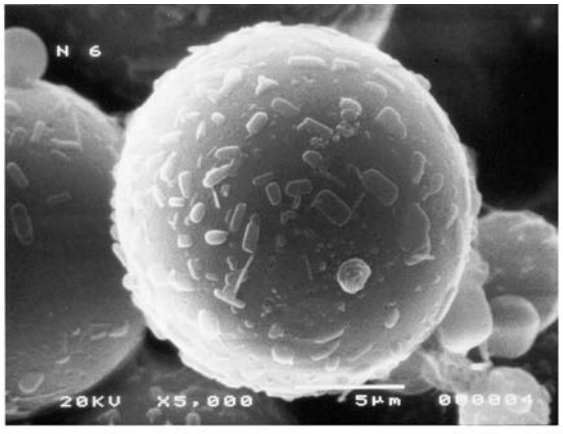
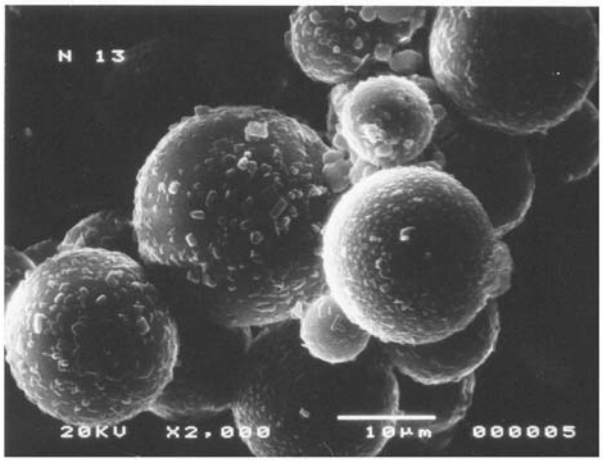
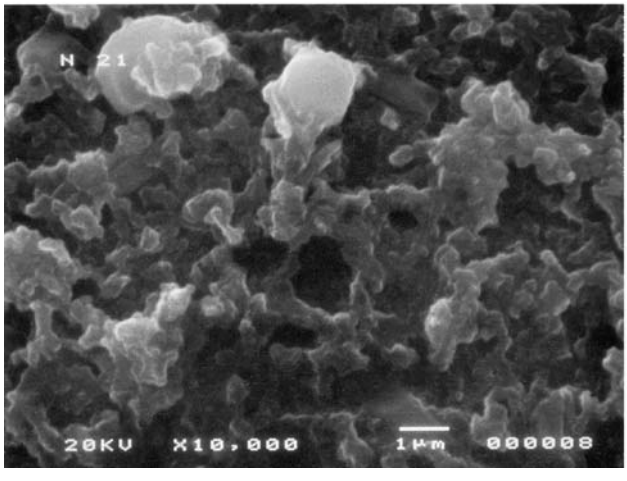
Fig. 2a–d Size distribution profiles for: **a** B_3 and B_4; **b** C_3 and C_4; **c** D_2, D_3 and D_4; **d** E_2, E_3 and E_4



a



b



c

Fig. 3a–c SEM micrographs for **a** B_3; **b** C_3; **c** B_3 outside surface

reactor, shape of stirring-rod). Obviously, under the same experimental conditions (stirring at 1000 rpm and the same reactor and stirring-rod), a different microparticle size can be obtained by using monomers with different chemical structures or by varying the degree of BSA derivatisation. In our experiments, a mean particle diameter of around 36 μm was obtained for samples B_3 and B_4 (Fig. 2a), 28 μm for C_3 and C_4 (Fig. 2b), 25 μm for samples D_2, D_3 and D_4 (Fig. 2c), and 22 μm for samples E_2, E_3 and E_4 (Fig. 2d). The results from dimensional analysis agree with the hypothesis that microparticles with more crosslinks show smaller dimensions.

In addition, using scanning electron microscopy we obtained information about the surface properties of the microparticles, and we were able to check that the microparticles were spherical. In Figs. 3a and 3b, the spherical shapes of sample B_3 and C_3, respectively, are evident. Figure 3c shows the surface of sample B_3, characterised by a high degree of porosity. Similar results were obtained for all of the spherical samples that we created.

Conclusions

BSA was successfully derivatised by reaction with MA, under mild conditions, in order to obtain a protein which contains chemical groups able to undergo radical polymerisation. Using different amounts of MA, several materials were prepared and the derivatisation degree was spectrophotometrically determined. All samples were water-soluble; we can suppose that the tertiary structure of the protein was practically unmodified. BSA_f and DMAA were employed for preparing crosslinked materials of different morphologies via radical polymerisation. By varying experimental parameters, including DD% of BSA_f and w/w ratio (BSA_f /DMAA), various protein microparticles with spherical geometries were realised. A decrease in concentration or in DD% of BSA_f led to less spherical geometries and more shape irregularity. Highly swollen particles (gelled) are usually obtained when the concentration and the DD% of BSA_f are extremely low.

The beads obtained showed a narrow size distribution profile and porous surfaces, as evidenced by scanning electron microscopy studies. The elevated water affinity and the high degree of swelling suggests that the proteins native structure is retained in these materials. Using comonomers with different chemical properties, the technique we have employed allows us to realise very versatile materials for biomedical and pharmaceutical applications.

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