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Albumin microspheres for intra-arterial tumour targeting. I. Pharmaceutical aspects

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Summary

Microspheres intended for intra-arterial targeting of cytotoxic agents have been prepared from human serum albumin using a water-in-oil emulsion technique with chemical cross-linking of the protein. The manufacturing variables affecting microsphere size, size distribution and stability have been examined as well as the phenomenon of swelling of freeze-dried microspheres in saline. In addition, the surface characteristics of the microspheres have been studied using microelectrophoresis and scanning electron microscopy.

Introduction

There is much current interest in the targeting of drugs to specific sites in the body (Gregoriadis, 1977). Targeting is intended to increase the specific distribution of a drug and hence, combined with lower dosing, to increase the therapeutic profile of a compound. Targetable drug delivery systems involve both drug–macromolecule and drug–antibody conjugates as well as particulate colloidal carriers. Fig. 1 illustrates the potential uses of colloidal particles for drug targeting (Tomlinson,

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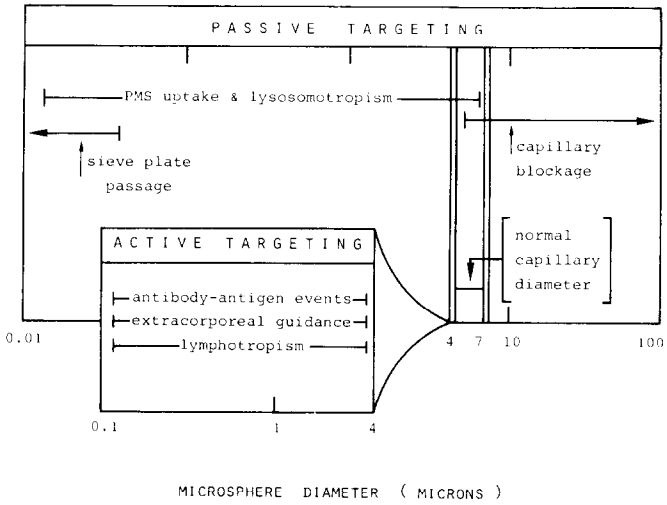

Fig. 1. Relation between microsphere size and biological targeting after intravascular injection.

1983). Four features are evident. (1) Intravenous, intra-arterial or intraperitoneal injection of particles of 0.1 to (approximately) 2 μm in diameter leads to a rapid clearance of particles from the blood stream by macrophages of the polymorphonuclear system (PMS). Particles of less than 50 nm can pass through the fenestrations of the liver endothelium and become localised in the spleen and bone marrow. (2) Intravenous delivery of particles above 7–12 μm leads to their mechanical filtration by the lungs, whereas particles between 3 and 12 μm will become entrapped within the capillary networks of the lung, liver and spleen (Kanke et al., 1980; Tomlinson et al., 1984). (3) Intra-arterial delivery of colloidal particles greater than 12 μm leads to their retention in the first capillary bed encountered. Such blockage can lead to "first-order" targeting of, for example, the liver or kidney and, for tumour-bearing organs, can lead to selective (i.e. "second-order") targeting to the tumour (Blanchard et al., 1965). (This latter effect is probably due to a qualitative and quantitative difference in capillary networks of the tumour compared to those of the host organ (Lindell et al., 1977).) Thus, a simple alteration in the size of microspheres and/or the route of administration leads to a manipulation of the targeting.

Fig. 1 further indicates that attempts are being made to alter the nature of colloidal particles in order to change these events. These alterations include: (a) the incorporation of magnetic particles in spheres of 1–2 μm in diameter so permitting extra-corporeal manoeuvering of the delivery systems with two-dimensional magnetic fields (Senyei et al., 1978; Widder et al., 1978); (b) alteration in surface charge (Illum and Davis, 1983); and (c) coating of the surface with either a bioadhesive (Goldberg et al., 1982), non-ionic surfactant, (Illum and Davis, 1983) or specific cell or tissue antibodies (Kaplan et al., 1983). Additionally, formulation of microspheres to alter their body distribution is also possible as shown by Sezaki and co-workers

(1982) using gelatin microsphere-in-oil "emulsions" for targeting to lymph nodes.

These techniques have merit and it is interesting to observe that frequently these novel delivery systems often act as probes of biological structures' function(s).

Microparticles are perceived as being important as drug carriers for targeting and controlled release, with currently over 20 different therapeutic roles being envisaged for them (Tomlinson, 1983). More than 25 materials have been studied for the microsphere matrix and to date more than 100 drugs have been incorporated into these carriers (Tomlinson, 1983). The literature and patent literature contain information on particular aspects of microsphere production and characteristics, though there exits no comprehensive, systematic study on the pharmaceutical and biopharmaceutical aspects of microspheres. In this present contribution we describe the pharmaceutical aspects of albumin microspheres intended for the intra-arterial delivery of cytotoxic compounds to tumours. Aspects covered include: preparative routes, sizing, size fractioning and distribution, stability, storage, colloidal characteristics (including electromobility) and presentation. In the following paper our studies on the release characteristics of model compounds from albumin microspheres of differing type are presented.

A preliminary report on these studies has been made (Tomlinson et al., 1982).

Experimental

Materials

Human serum albumin (HSA) was Cohn Fraction V grade obtained from SERVA (Heidelberg, F.R.G.). Tween 80 and highly refined olive oil were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium cromoglycate (SCG) was of pharmacopoeial grade and was a gift from Fisons Limited, Loughborough, U.K.

Methods

(1) Preparation

Human serum albumin microspheres in the range 0.4–40 μm have been prepared using the manufacturing procedures outlined below. To a flat-bottomed glass beaker (diameter 60 mm, height 110 mm) equipped with four baffles (4 mm depth) positioned against the wall of the beaker were added 125 ml of olive oil. A motor-driven four-bladed axial-flow impeller was placed in the centre of the beaker with a 3-mm distance between the baffles and the impeller blade. The impeller was submerged two thirds into the oil. After pre-stirring the oil for 30 min at the desired speed, an aqueous solution of HSA in isotonic phosphate buffer (pH 7) was added dropwise from a syringe to the olive oil. The resulting water-in-oil emulsion was stirred for an additional period of time depending upon the method of stabilization chosen.

(i) Non-stabilized microspheres. These were prepared at room temperature (20 $\pm$ 3°C) by addition of 0.5 ml of a 25% (w/v) HSA solution to 125 ml of oil. After stirring for 90 min, 60 ml of diethyl ether were added to the water-in-oil emulsion.

Stirring was continued for a further 10 min after which the formed microspheres were isolated by centrifugation for 5 min at 2000 rpm followed by decantation of the supernate. After resuspending the microspheres in diethyl ether and collecting them on a 0.8 μm polycarbonate filter, residual oil was removed by further washing with diethyl ether.

(ii) Stabilization with 2,3-butadione. After stirring a water-in-oil emulsion produced upon mixing 0.5 ml of a 25% (w/v) aqueous HSA solution with 125 ml of oil for 90 min at the desired stirring speed, the emulsion was transferred to a sealed beaker so that stirring could continue without evaporation of the 2,3-butadione. Stirring was continued at 40 rpm using a magnetic stirrer. 2,3-Butadione was then added in either a 3 ml or a 15 ml volume to produce a final 2,3-butadione concentration of 0.27 $mol \cdot dm^{-3}$ or 1.22 $mol \cdot dm^{-3}$. After reaction for a desired period, the microspheres were centrifuged, washed and collected as above. 2,3-butadione cross-linked microspheres were also prepared by suspending 40 mg of non-stabilized microspheres in 100 ml of diethyl ether containing various concentrations of cross-linker.

(iii) Stabilization with glutaraldehyde. To 125 ml of oil were added 0.4 ml of a 25% (w/v) aqueous HSA solution. After stirring for 15 min, 0.1 ml of an aqueous glutaraldehyde solution were added to produce a final (aqueous) glutaraldehyde concentration of between 1 and 5%. Stirring was continued at the desired rate and for the desired time, after which the microspheres were collected and washed as described previously.

(iv) Heat denaturation. Human serum albumin microspheres were also prepared using a heat denaturation method. The method was similar to that described above for non-stabilized microspheres, except that the temperature of the oil was at 125°C with denaturation times of between 10 and 60 min being used.

(2) Lyophilization and storage

After collection, microspheres prepared via these routes were lyophilized using an FTS Systems apparatus at 10–20 mTorr and −60°C for 18 h. Upon freeze-drying the sample, vials were sealed with an air-tight seal and stored at 4°C in the dark.

(3) Fractionation

Freeze-dried microspheres were fractionated by sieving using microsieves obtained from Veco (Eerbeek, The Netherlands). Approximately 80 mg of freeze-dried microspheres were suspended in 50 ml of chloroform, ultrasonicated for 5 min and then sieved between the appropriate size microsieves. After sieving, the microspheres were again freeze-dried and stored as before.

(4) Sizing

Microspheres were generally sized in a 0.1% Tween 80 saline solution using a Coulter Counter (model Z_B) equipped with a Channelyser (Type C-1000) (Coulter Electronics, Dunstable, U.K.). Some microspheres were also sized using a Scanning Electron Microscope (Cambridge M2A). These specimens were sputter-coated with a 35 nm layer of Au/Pd. In addition, it was found convenient to carry out the

measurement of the diameter with a light-microscope equipped with a calibrated micrometer; calibration was performed with latex particles of known diameter.

(5) Microelectrophoresis

Microelectrophoretic characteristics of various drug- and non-drug-containing microspheres were measured at 25°C using a particle microelectrophoresis apparatus (Mark II, Rank, Bottisham, Cambridge, U.K.). Microspheres were prepared with and without sodium cromoglycate (a hydrophobic dianion) by cross-lining the microspheres with 5% glutaraldehyde for 4 h and using a mix-cell stirring speed of 825 rpm to produce microspheres of diameter between 12.7 and 28.2 μm, which were then sieved (in chloroform) between 15 and 25 μm. These microspheres were examined either without pre-ultrasonication or with an ultrasonication step. In this latter case, approximately 40 mg of freeze-dried microspheres were suspended in 15 ml of 0.1% Tween 80 phosphate buffer (pH 7) and ultrasonicated for 5 min followed by filtration and washing with water, ethanol and diethyl ether. This procedure was necessary to avoid a large release of cromoglycate from the microspheres upon subsequent suspension in saline. Zeta potentials were measured in three media: (1) 0.1% Tween 80 physiological salt solution; (2) 0.1% Tween 80 plasma; and (3) plasma.

Results and Discussion

Although there exist a number of patents on the manufacture of albumin microspheres for drug and diagnostic use (e.g. Yapel, 1979), there are few reports in the literature on the pharmaceutical aspects of albumin microsphere production. Table 1 gives these aspects as they relate to product manufacture and presentation.

There are two types of microsphere production. The first involves either thermal denaturation at elevated temperatures (95–170°C) or chemical cross-linking in vegetable oil or isooctane emulsions. The latter method, which is claimed to produce

TABLE 1

PHARMACEUTICAL AND BIOPHARMACEUTICAL CONSIDERATIONS IN THE DEVELOPMENT OF A MICROSPHERE PRODUCT

(1) Core material
(2) Route of preparation with respect to (3–8)
(3) Size
(4) Drug incorporation
(5) Type and amount of drug
(6) Drug release (in vitro and in vivo)
(7) Drug stability during (2) and (9)
(8) Microsphere stability (in vitro and in vivo)
(9) Storage
(10) Surface properties
(11) Presentation (e.g. free-flowing, freeze-dried powder)

Fig. 2

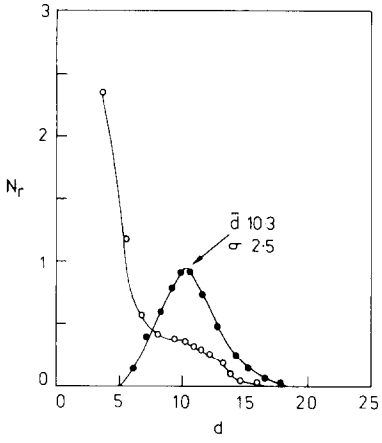

Fig. 3

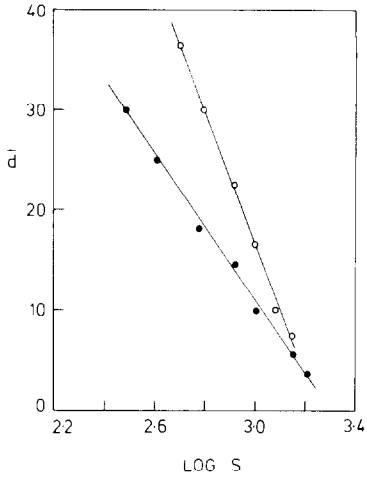

Fig. 2. Relative number of 2,3-butadione stabilized albumin microspheres per milligram ($N_r \times 10^{-3}$) versus sphere diameter ($\bar{d}$, μm), determined using a Coulter Counter (model Z_B) equipped with a Channelyser. Open and closed datum points refer to spheres produced in a non-baffled and baffled cell, respectively. (Manufacture was at 1120 rpm).

Fig. 3. Relationship between mean diameter of albumin microspheres ($\bar{d}$, μm) and the stirring speed of the impeller (S, rpm). Open and closed datum points refer to spheres manufactured using highly refined and contaminated olive oil, respectively.

"hydrophilic" microspheres, depends upon chemical cross-linking of microspheres in a water-in-oil emulsion using concentrated polymer solutions as the dispersing phase (Longo et al., 1982). Second, albumin microspheres (for diagnostic use) may be prepared using either a simple one-step preparative method involving thermal denaturation of protein aerosol in a gas medium (Przyborowski et al., 1982) or an aerosol step followed by denaturation in oil (Millar et al., 1982). In this present study we chose to manufacture microspheres using a water-in-oil method coupled with chemical cross-linking of the protein, since initial studies with thermal denaturation techniques (Experimental) resulted in decomposition of the cytotoxic drugs under study. Although most studies have used cottonseed oil as the continuous phase in manufacture, for its purity we have chosen highly refined olive oil as the continuous phase.

It has been found using the emulsion/chemical cross-linking technique, that the design of the mix-cell is critical to produce a uniform and reproducible product. Fig. 2 shows that the manufacture using a flat beaker as mix-cell gave microspheres having a broad size range with no detectable maximum, whereas manufacture in a mix-cell fitted with baffles gave well-defined microspheres having a distinct maximum. The most critical factor controlling the mean diameter was found to be the rate of stirring of the impeller.

Fig. 3 gives the relationship found between the mean microsphere diameter and the impeller stirring speed and shows that as the speed is increased, the mean

diameter of microspheres is reduced. It has been found that the grade of olive oil affects the diameter of the formed microspheres. Fig. 3 gives results obtained using a different type of olive oil (Brocacef, Maarssen, The Netherlands). Although this led to the production of well-defined microspheres, it was discontinued from use since, without the addition of a cross-linking agent, it resulted in stable microspheres having a slow drug release (presumably due to an oil contaminant which was cross-linking the microspheres). Cross-linking was found to have no detectable influence on the mean diameter or size distribution of the microspheres. Microspheres are formed very rapidly in the water-in-oil emulsion and this enabled the stirring speed of the impeller to be reduced after sphere formation, since this had no subsequent effect on microsphere diameter. This was found to be useful during cross-linking with 2,3-butadione, which rapidly evaporated at high stirring speeds.

Although stirring speed affords a simple way in which mean microsphere diameter may be controlled, it is preferable to have narrower size fractions of microspheres available for therapeutic use. This is easily accomplished using microsieves, as described in the Experimental section. Fig. 4 gives the size distribution for some sieved human serum albumin microspheres, and shows that an extremely narrow and well-defined size fraction of microspheres can be produced. Since microspheres are readily suspended in chloroform, sieving is rapid. For water-soluble drugs, using chloroform as the suspending medium, sieving does not result in a loss of drug. The relationship between the mean diameter of sieved microspheres ($\bar{d}$) and the total number of microspheres per milligram freeze-dried weight of sphere (N_t) may be given by: $N_t = \log \bar{d}$ ($n = 5$; $r = 0.9$).

This study has also shown that albumin microspheres may be produced without

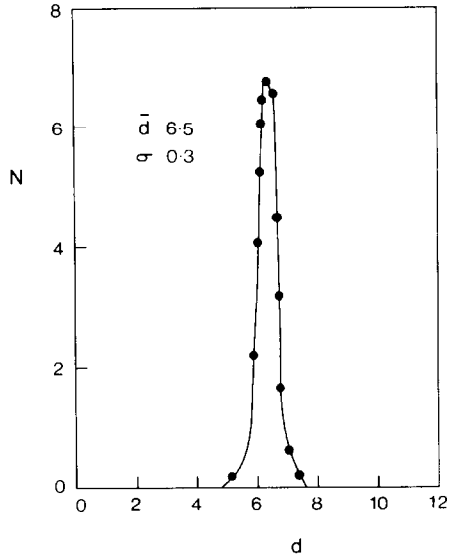

Fig. 4. Size distribution of butadione-stabilized albumin microspheres sieved between 5 and 10 μm. The plot is of the number of microspheres per milligram ($N \times 10^{-4}$) versus sphere diameter ($\bar{d}$, μm).

recourse to either chemical cross-linking or heat denaturation. Thus, at room temperature "non-stabilized" microspheres may be formed, collected and lyophilized (see Experimental). The feature of these spheres is that they dissolve completely within 2–5 min in phosphate buffer (pH 7). This affords a rapid method for readily estimating the extent of drug incorporation. Such non-stabilized microspheres are obtained if, instead of a cross-linking agent, diethyl ether is added to the water-in-oil emulsion. Widder et al. (1978) have stated that non-stabilized microspheres 1–2 μm in diameter may be stabilized upon suspension in diethyl ether containing 0.2 mol · dm^{-3} butadione. Using a similar approach, we have found that microspheres in the range 15–25 μm in diameter require a much longer time for stabilization than do microspheres of 1–2 μm in diameter. Using 0.2 mol · dm^{-3} butadione, stabilization of the smaller microspheres required 10 min, whereas periods in excess of 2 h were necessary to stabilize larger microspheres. Post-microsphere stabilization with butadione had to be performed most carefully. For 15–25 μm diameter microspheres, a too strong agitation of non-stabilized microspheres resulted in the formation of small fragments of denatured albumin. Raising the concentration of butadione helps to reduce stabilization times and consequently to reduce the likelihood of sphere disruption.

The recovery of albumin microspheres after production, collection and lyophilization in the absence or presence of drug was approximately 96%. Freeze-drying of air-dried microspheres in the 15–25 μm range resulted in a 9.7% loss in their weight. Exposure of these lyophilized microspheres (which contained approximately 17% by weight of sodium cromoglycate and had been prepared using cross-linking with glutaraldehyde for 18 h) to the atmosphere led to a 3.2% increase in their weight after 1 h, an increase of 6.8% after 5 h and after 24 h and further to an increase in weight of 9.2%.

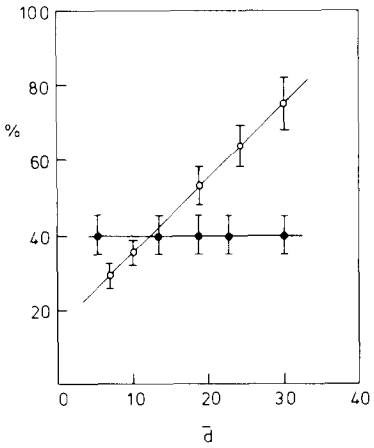

Fig. 5. Relationship between mean diameter of freeze-dried albumin microspheres ($\bar{d}$, μm) and extent of swelling (%). Open and closed datum points refer to microspheres containing 17% SCG by weight and non-drug-bearing microspheres, respectively.

Upon being placed into a 0.1% Tween 80 saline solution at 20°C, freeze-dried, non-drug-bearing albumin microspheres prepared by chemical cross-linking were observed to swell. Such swelling is complete within 2 min and is uninfluenced by the extent of cross-linking and/or the size of the spheres. Fig. 5 shows that for microspheres with diameters in the range 5–60 μm, the increase due to swelling is approximately 40%.

Zolle et al. (1970) have reported that in normal saline heat-denatured albumin microspheres showed a 50.5% average increase in diameter after 5 min, with less swelling being observed with microspheres prepared at higher temperatures. In addition, these workers reported a case where further swelling occurred after an additional period of 1 h. Similar observations have been reported in the literature for thermal-denatured albumin microspheres (Widder et al., 1978). Since albumin microspheres from this present study do not swell in chloroform, sieving of lyophilized microspheres followed by suspension in saline leads to an increase in the mean diameter of the microspheres. The incorporation of highly water-soluble drugs into the microspheres can lead to a greater extent of swelling. For example, 30-μm lyophilized microspheres containing approximately 17% by weight of the dianionic drug sodium cromoglycate (SCG) and prepared using cross-linking with glutaraldehyde for 18 h, increased in diameter on average by 75% when placed in saline, whereas similar non-drug-bearing microspheres increased in diameter by only approximately 40%. Fig. 5 also gives the relationship found between the size of freeze-dried SCG-bearing microspheres and the extent of swelling. This shows that the larger the microspheres, the greater is the percentage swelling. These effects are obviously of therapeutic relevance for those cases where microsphere size is critical.

The effects for other manufacturing factors on the size of microspheres are given in Table 2. It is clearly shown that alteration of the size of the dispersed phase during manufacture leads to a corresponding alteration in the size of the formed microspheres. Interestingly, although most methods for producing microspheres use

TABLE 2

EFFECT OF MANUFACTURING VARIABLE ON THE MEAN DIAMETER OF HUMAN SERUM ALBUMIN MICROSPHERES [a]

Factor	Change	Mean diameter [b]
oil viscosity [c]	decrease	increase
oil amount	increase	increase
protein amount	increase	increase
aqueous phase [d]	increase	increase
stirring speed	increase	decrease
surfactant [e]	addition	no effect

[a] Standard factors were 125 ml olive oil and 0.5 ml of a 25% w/v HSA solution.

[b] Estimated using microscopy.

[c] The viscosity of the oil was varied by addition of petroleum ether 100–140 to give an end volume of 125 ml.

[d] Addition of 1 ml of a 12.5% w/v HSA solution.

[e] Addition of 0.1% sodium dodecylsulphate to the aqueous HSA solution.

water-in-cottonseed oil emulsions and an ionic surfactant in the manufacture, here this had no discernible effect on microsphere diameter when olive oil was used as the continuous phase.

The colloidal and surface characteristics of a microsphere delivery system are of importance to its presentation, injection and biofate. The present microspheres are designed to be presented for clinical use as a free-flowing, lyophilized drug-containing sterile powder, which is to be suspended in a sterile solution of 0.1% Tween 80 in isotonic salt solution. Although for the intra-arterial delivery of microspheres uptake by the polymorphonuclear system is unlikely, it is of some interest to us to know what the electrophoretic mobilities of drug-containing microspheres are, since this can influence platelet adhesion (Muramatsu et al., 1982), and (for microspheres of less than 2 μm in diameter) the manner of recognition and excretion by the PMS (Wilkins and Meyers, 1966). Table 3 gives the measured zeta potentials for a number of different microsphere systems in various media. It is shown that drug-bearing and non-drug-bearing albumin microspheres prepared in this study are negatively charged and that the magnitude of the zeta potential is most dependent upon both the media and the presence of (ionized) drug. In plasma, with and without the presence of 0.1% Tween 80, all microspheres studied have a fairly similar zeta potential ranging from 5.3 to 9.5 mV (at 25°C). However, in physiological salt solution observed zeta potentials were much higher (8.7–18.9 mV). Strikingly, although microspheres containing sodium cromoglycate had a high zeta potential after ultrasonication (and irrespective of the presence or absence of nonionic surfactant), cromoglycate containing microspheres which had not been subject to ultrasonication, had a much lower zeta potential value. The data given in Table 3 suggest that upon ultrasonication a surface layer of cromoglycate is removed, which gives the microphores the same characteristics as non-drug-bearing microspheres and that in plasma, adsorption of plasma components tends to give all types of microspheres comparable

TABLE 3

ZETA POTENTIALS OF HUMAN SERUM ALBUMIN MICROSPHERES [a] AT 25°C

Microsphere	Zeta potential at 25°C (mV)			Mean diameter (μm)
	Suspension medium			
	0.1% Tween 80 in physiological salt solution	0.1% Tween 80 in plasma	Plasma	
0.1 mol·dm^{-3} SCG; ultrasonicated [b]	−18.5	−9.5	−8.9	21.7
0.1 mol·dm^{-2} SCG non-drug containing; ultrasonicated [b]	−8.7	−7.2	−6.9	23.0
non-drug-containing	−18.9	−7.1	−8.2	21.7
	−13.0	−5.3	−7.5	21.7

[a] Stabilized by cross-linking for 4 h with 5% glutaraldehyde.

[b] Approximately 40 mg freeze-dried microspheres suspended in 15 ml 0.1% Tween 80 physiological salt solution, followed by ultrasonication for 5 min, filtration, washing with water, ethanol and ether, and then freeze-drying.

electrophoretic mobility, which is similar to that described recently by Muramatsu et al. (1982) for plasma component adhesion to poly(1,4-piperazinediylterephthaloyl) microcapsules.

Scanning electron microscopic photography of microspheres shows that surfaces are relatively smooth, though with some there are surface crenellations.

Concluding remarks

Some of the pharmaceutical aspects of the production of human serum albumin microspheres intended for intra-arterial drug targeting to tumours have been examined. It has been demonstrated that well-defined microspheres may be easily produced, and that their size and surface characteristics can be changed by adjusting a number of manufacturing and post-manufacturing variables. In a following paper the influence of a number of these manufacturing variables and microsphere characteristics on drug incorporation and drug release from human serum albumin microspheres will be described.

A feature not reported here is the sterilization and validation of the sterilization of drug-containing albumin microspheres. Oppenheim (1981) has suggested that the presence of glutaraldehyde in the water-in-oil emulsion renders microspheres sterile, whereas microspheres used for diagnostic purposes are sterilized by either heat or by gamma irradiation. Since both of these latter factors will affect microsphere stability (and hence drug release), our current studies are directed towards the production of drug-containing human serum albumin microspheres using aseptic techniques.

Hopwood et al. (1970) have suggested that upon interaction with glutaraldehyde proteins are not denatured to any marked extent, but that glutaraldehyde–protein links form at the rate of $1\ s^{-1} \cdot mol^{-1} \cdot dm^{-3}$ with the rate of reaction increasing with pH. Further, amongst the basic amino acids in the protein, only lysine combines with the glutaraldehyde. Lee et al. (1981) have found that as the concentration of glutaraldehyde in the emulsion was 1, 2, 3 or 4% then the number of modified lysine residues increased to 21, 33, 44 and 47, respectively. These workers also found that, although the rate of drug release could be controlled by varying the density of the protein microsphere matrix, only microspheres cross-linked with 1% glutaraldehyde were susceptible to digestion by chymotrypsin (as indicated by their biodegradation characteristics).

These considerations will be further discussed in a following paper on drug incorporation and drug release.

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