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Novel approach for preparing nontoxic stealth microspheres for drug delivery

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ABSTRACT

Polyethylene glycol 2000 (PEG) entrapped, bovine serum albumin (BSA) microspheres were prepared and optimized. These microspheres were then analyzed to understand the stealth effect to the microspheres imparted by incorporated polyethylene glycol (PEG). Microsphere preparations (formulations) were studied for particle size, zeta potential, free BSA content, and in-vitro uptake by human micro-vascular endothelial cells (HMECs). The results demonstrate, a formulation containing 37.5 % PEG (w/w) exhibits an acceptable particle size of <5 μm, good suspendibility with zeta potential of -35 mV, and confers some degree of stealth property to the microspheres. These non-toxic, biodegradable and long circulating microspheres have the potential to be used for extended intravenous (IV) drug delivery. Statistical analysis done by ANOVA and results with $p<0.05$ were considered significant.

1. Introduction

Cross-linked albumin microspheres are a potential delivery method for slow release of drugs or antigens to the targeted sites [1-7]. Bovine serum albumin microspheres have been extensively investigated in controlled release drug delivery systems since introduced by Kramer [8]. Release of a drug from the albumin microspheres can be manipulated using the amount of BSA, size and suspendibility of the microspheres, and most effectively, the degree of cross-linking [9]. Earlier methods of surface cross-linking could control the release as long as the surface was intact. Enzymes and other in-vivo factors quickly overcome the surface barrier. The more recently developed matrix cross linking has an added advantage where cross-linkages among the $-NH_2$ (amine) groups takes place in the entire matrix, that allows drugs to slowly release in the surrounding environment. These microspheres are easily phagocytosed and act as an excellent delivery vehicle for intercellular delivery. However, for a systemic drug delivery, longer circulating microspheres are required. Polyethylene glycol has demonstrated imparting the stealth property by avoiding opsonization in various studies pertaining to liposomal preparations [10-12] and drug molecules [13]. PEG is non-toxic and, after cleavage in vivo from the PEG-drug conjugate, free PEG cleared rapidly out of the body by the kidneys. The stealth effect was achieved by covalently linking PEG to the drug molecules [14,15]. However, due to large size of modified drug–PEG molecules, they have difficulty in crossing the cell membrane, hence this approach is not advantageous. On the other hand, if a delivery vehicle (e.g., microspheres) can be made stealth with drug inside them, then drug can be released for a longer period of time in the circulation. There are no reports of evaluating stealth effect of PEG on BSA microspheres. One approach of covalently linking the PEG to BSA and subsequently making microspheres was likely to constitute stealth microspheres [16]. However, these microspheres rapidly disintegrate in the solution and do not provide an appropriate vehicle for sustained drug delivery.

In the present study, we explore the possibility of providing stealth property to the microspheres by incorporating PEG 2000 (known for prolonging plasma residence time of the liposomal formulations [17]) into the BSA prior to cross-linking of the BSA matrix. The purpose is to entrap PEG without covalently binding to the drug molecules or BSA. This is accomplished by cross-linking BSA in the presence of PEG and subsequently obtaining microspheres by a spray drying process. Different concentrations of PEG were tested and investigated for free BSA present in the microspheres to optimize the process. Microspheres were analyzed and characterized by particle size determination, zeta potential measurement, infra–red spectroscopy and differential scanning calorimetry. Surface characteristics of microspheres were studied by electron microscopy. For investigating the stealth property we employed vascular endothelial cells, which are the first one to come in contact with microspheres when delivered intravascularly. In the present study, we considered Human Micro-vascular Endothelial Cells (HMECs), a known stable phagocytic cell line, [18] to determine the stealth effect of PEG present in the microspheres.

2. Experimental

2.1. Materials

Bovine serum albumin, polyethylene glycol 2000 and glutraldehyde (GA) were obtained from Sigma (St. Louis, MO).

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HMECs and the nutrient media [MCDB 131, supplemented (EBM)] were obtained from CDC (Atlanta, GA). HMECs were grown in 75 cm^2 flasks, subcultured in 24-well tissue culture plates obtained from Fisher Scientific, and incubated at 37 °C in the presence of 5% CO_2 for 24 hrs (80-90% confluence).

2.2. Methods

2.2.1. Preparation of microspheres

Seven formulations (A to G, Table 1) containing different amounts of PEG ranging from 0.0–66.7% (w/w, relative to BSA) were prepared in 5% BSA (w/v) and cross-linked by glutraldehyde for 18 hrs at room temperature as described previously [1-7]. Excess glutraldehyde was neutralized with sodium bisulfite. Cross-linked solutions were spray-dried using a Büchi mini spray dryer (Büchi 191). Microspheres were aspirated through a chamber where they were cooled down to a lower outlet temperature and deposited into a product container through a cyclone. The prepared microspheres were collected and refrigerated at 5 °C until further use.

Table 1. Free BSA amount in cross-linked microspheres containing PEG. Formulations A, B, C, D, E, F, and G were prepared with the addition of 0.00, 9.09, 16.67, 28.57, 37.50, 50.00 and 66.67 % of PEG (w/w with respect to BSA) in 5% (w/v) BSA aqueous solution.

Formulations	% PEG	% BSA	% of free BSA
A	0.0	100.0	17.8
B	9.1	90.9	17.3
C	16.7	83.3	12.6
D	28.6	71.4	16.9
E	37.5	62.5	16.8
F	50.0	50.0	91.8
G	66.7	33.3	106.6

2.2.2. Analysis of free BSA

The purpose of this study was to evaluate the effect of PEG on the cross-linking of BSA in the solution. Free BSA which could not be cross-linked due to the presence of PEG remained soluble in water and was quantified by a spectrophotometer. Microspheres equivalent to 20 mg of BSA content were suspended in 10 ml of deionized water at 37 °C for 1 hr, vortexed and centrifuged for 15 minutes at 3000 rpm. Samples of 5 mL clear supernatant were taken and absorbance was recorded by a Perkin Elmer Spectrophotometer at the 277 nm wavelength. Standard solutions of BSA with concentrations ranging from 0.032 mg/mL-4.000 mg/mL of BSA in deionized water were freshly prepared and also read at the 277 nm wavelength. The amount of free BSA present in the microsphere formulations was calculated.

2.3. Physical characterizations

2.3.1. Particle size

The size of the microspheres plays a significant role in the uptake by phagocytes and delivery of drugs. Particle size analysis was performed using a Spectrex Laser Particle Counter, PS-2000. Approximately 0.5 mg of microspheres of each formulation were suspended in 7 mL of filtered deionized water in separate 10 mL vials. The vials were vortexed for 10 seconds and placed in the laser particle counter to measure the particle size.

2.3.2. Zeta potential

Zeta potential determined in phosphate buffer saline (PBS) of pH = 7.4 (PBS) by using a Malvern Zeta-sizer.

2.3.3. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR was used to identify and determine the characteristic functional groups in PEG, BSA and microspheres and experiments were carried out using standard protocol of Perkin Elmer FT-IR. We used FT-IR to identify PEG in the microspheres and to investigate if there was interaction between PEG and BSA. A small amount of BSA, PEG and microsphere formulation (all individually) was placed on the FTIR crystal and the spectrum recorded from 500-4000 wave numbers (cm^{-1}).

2.3.4. Differential scanning calorimetry (DSC)

The DSC was used to determine the compatibility of excepients. It is also used to notice heat induced changes in the polymer. We employed this tool to determine if PEG affects the cross-linking of BSA. DSC was performed in DSC Q 100 by weighing ~5 mg of each of the microsphere formulations in standard aluminum pans which were non-hermetically crimp closed using TA's blue DSC sample press. The encased sample and reference pan were placed on the heating stages in the instrument and were heated from 27.5 °C to 325°C with a ramp speed of 5 °C per minute.

2.3.5. Scanning electron microscopy (SEM)

The SEM was carried out at Centers for Disease Control (CDC, Atlanta, GA) using an Environmental Scanning Electron Microscope XL 30 ESEM manufactured by the FEI Company, Hillsboro, Oregon. This Environmental Scanning Electron Microscope achieves high magnification with excellent resolution. Microsphere samples were placed on holders and coated with gold in Sputter Coater to achieve a thickness of 2 nm. Coated samples were placed in the ESEM chamber and vacuum was applied. Pictures were taken at around 40,000x magnifications to visualize the surface characteristics of microspheres.

2.3.6. In-vitro cell uptake study

HME cells were grown and maintained in MCDB 131 complete medium at 37 °C in the presence of 5% carbon dioxide. Microspheres were labelled with fluorescamine dye [19,20] as per standard product information sheet F9015 provided by Sigma (Milwaukee, WI). Labeled microspheres were added to the HME cells at a ratio of 10:1 and the cell suspension was evenly made in the culture plates. At various time points (0.5-48 hrs) cells were washed to remove unattached and unphagocytosed microspheres. HME cells were observed under fluorescence microscopy to determine the microspheres uptake. HME cells were lysed with triton-X and fluorescence was measured by using Tecan Genius fluorometer at the excitation wavelength of 485 nm and the emission wavelength of 535 nm and the percent uptake of microspheres was calculated.

3. Results and discussion

3.1. Free BSA analysis

Table 1 shows formulations F and G containing high PEG concentrations of 50.0% and 66.7% (w/w), respectively, resulting in high amounts of free BSA in the microspheres. These microsphere formulations were fragile and disintegrated in water very easily, forming an almost clear solution within a few minutes.

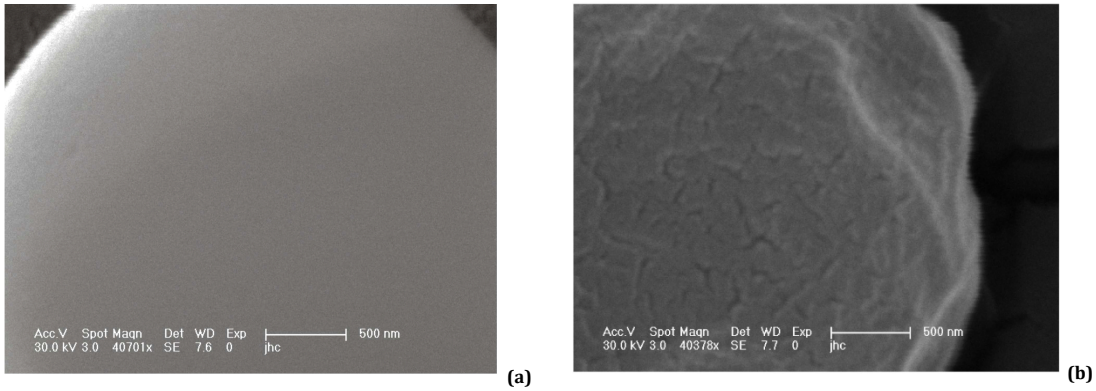

Figure 1. SEM of microsphere formulations showing surface characteristics of cross-linked BSA microspheres at magnification of x 40,000; (a) Formulation A, BSA microspheres without PEG 2000 and (b) Formulation E, BSA microspheres with PEG 2000.

This phenomenon could be due to the interference by a high amount of PEG hindering the cross-linking of BSA resulting in no or minute cross-linking among the BSA molecules, hence a high yield of free BSA in the formulation. We noticed the percent recovery exceeds 100% for formulation G, which means the sample weight taken equivalent to 20 mg of BSA actually contains more BSA than anticipated. This could be due to the PEG containing a very high number of associated water molecules (hydration number 136±4) and loss of PEG (and associated water) during the spray drying process, resulting in more BSA in the final product than estimated. Data indicates PEG content higher than 37.5 % is unsuitable, as it interferes with cross-linking and may result in burst release. Based upon this data, formulation F and G were found unsuitable for our study and further analysis not performed.

3.2. Particle size and zeta potential

Table 2 shows formulations A to E have a mean particle size of <5 μm and are ideal for encapsulation and drug delivery, while Table 3 shows that zeta potential is high and results in good dispersion of the microspheres in PBS. This implies the microspheres will remain in suspension for a long time and with low chance of forming aggregates and potentially clogging the capillaries.

Table 2. Particle size of various formulations evaluated.

Measurements	Formulations				
	A	B	C	D	E
1	3.11	3.60	3.74	3.80	3.66
2	3.05	3.13	3.50	3.71	3.13
3	3.03	3.07	3.46	3.57	3.07
Average size (μm)	3.06	3.48	3.57	3.69	3.29
SD	0.04	0.29	0.15	0.12	0.32

Table 3. Zeta potential of the formulations.

Measurement	Formulations				
	A (mV)	B (mV)	C (mV)	D (mV)	E (mV)
1	-34.23	-31.79	-32.73	-31.89	-33.27
2	-33.83	-30.39	-31.83	-34.03	-37.79
3	-38.46	-30.92	-33.86	-34.59	-35.91
Average	-35.51	-31.03	-32.81	-33.50	-35.66
SD	2.57	0.71	1.02	1.42	2.27

3.3. Scanning electron microscopy

The Figure 1 shows the SEM images of the microspheres and highlights the surface characteristics. The BSA microspheres without PEG 2000 (Figure 1a) show smooth topography whereas BSA microspheres with PEG 2000 show a wrinkled topography; (Figure 1b). This is indicative of the fact that PEG 2000 is exposed at the surface of the microspheres that when comes in contact with water, will form hydration layer around the microspheres, thus imparting the desired stealth property.

3.4. Differential scanning calorimetry

The DSC showed no variability in the melting curve of BSA in the formulations (Figure 2). Although it is obvious that PEG has no cross-linking sites, DSC further confirmed that PEG had no chemical interactions with BSA or to itself by the cross-linker (GA).

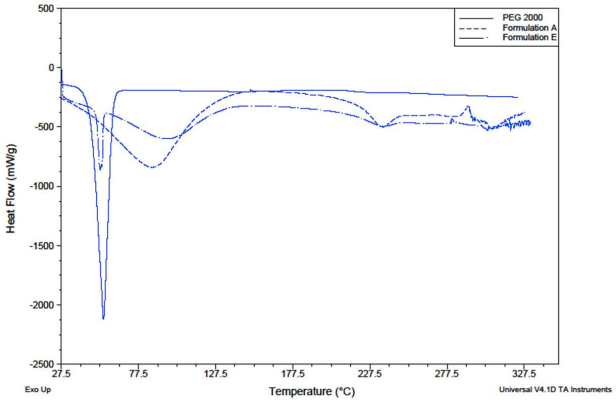

Figure 2. DSC profile of the PEG 2000, formulation A (BSA only microspheres) and formulation E (BSA microspheres with PEG 2000).

3.5. Fourier transform infrared spectroscopy

FT-IR of the formulations indicate that the BSA and PEG are chemically compatible, and the presence of PEG does not alter the course of BSA cross-linking. FTIR spectroscopy is a powerful analytical tool, able to quickly identify complex molecules bearing a rich assortment of IR bands. It is interesting to note that formulation A (cross-linked BSA with no PEG) shows very strong carbonyl and amine group peaks at 1532 cm^{-1} and 1645 cm^{-1}, respectively (Figure 3). These strong peaks are due to the presence of encapsulating matrix BSA that is a large protein containing great number of amine and carbonyl groups. Similarly formulation E, that compose of PEG modified cross-linked BSA encapsulating material, not only shows the characteristic peaks of BSA (*vide supra*) but also contains a strong absorption band at 1106 cm^{-1} due to ether

Table 4. Percent uptake of various microsphere formulations by HMECs shows the comparative uptake of formulations A, B, C, D, and E containing 0.00, 9.09, 16.67, 28.57, and 37.50% PEG (w/w) in 5% BSA solution, respectively.

Percent Uptake of Microspheres									
Formulations	Time in hours								p-value
	0.5	1	2	4	8	12	24	48	<0.05
A	7.0%	7.8%	8.2%	8.2%	8.3%	8.4%	8.4%	8.4%	0.73
B	6.9%	7.9%	8.0%	8.1%	8.3%	8.3%	8.3%	8.2%	0.75
C	7.1%	7.6%	8.0%	8.1%	8.2%	8.3%	8.3%	8.2%	0.59
D	6.4%	7.6%	7.7%	7.6%	8.1%	8.3%	8.3%	8.2%	0.28
E	3.9%	3.9%	3.8%	3.8%	3.8%	3.7%	3.9%	3.9%	0.00

group present in PEG and a strong absorption band at 2874 cm^{-1} indicating -O-CH_2-CH_2-O- linkage (ethylenedioxy) of PEG (Figure 4).

3.6. Uptake of microspheres from various formulations by HME cells

The results of the microspheres uptake by HMECs are shown in Table 4. Data indicates, microspheres from formulation E with PEG concentration of 37.5 % were least engulfed by HME cells compared to the other microsphere formulations containing PEG. This difference in phagocytosis is statistically significant when compared to formulation A, which has no PEG. (ANOVA at p <0.05)

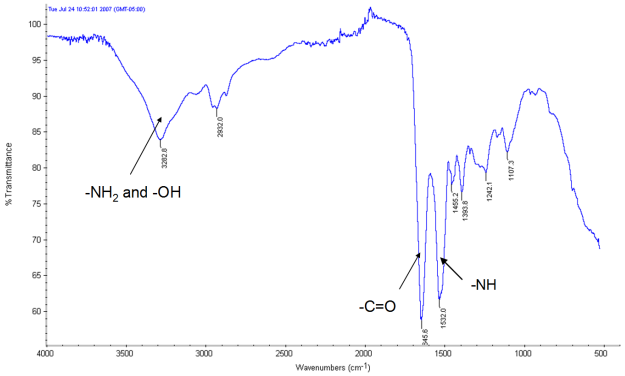

Figure 3. IR spectrum of cross-linked BSA microspheres. Arrows indicate absorption at 1645 cm^{-1} and at 3282 cm^{-1} from carbonyl group and amine and hydroxyl groups, respectively.

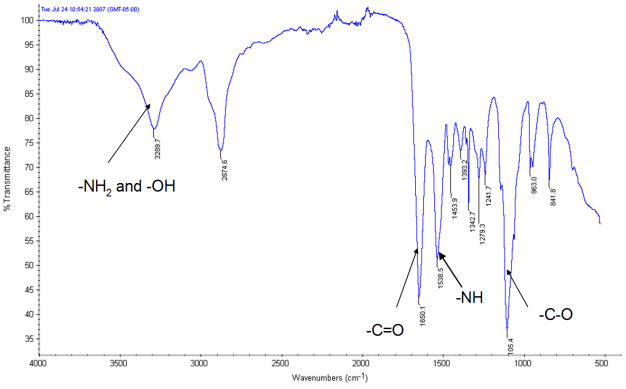

Figure 4. IR spectrum of cross-linked BSA microspheres with PEG. Arrows indicate absorption at 1105 cm^{-1}, 1650 cm^{-1} and at 3282 cm^{-1} from ether group, carbonyl group and amine and hydroxyl groups, respectively.

4. Discussion

PEG is a non-toxic pharmaceutical ingredient and is readily removed by the kidneys. Addition of PEG, at 37.5 % (w/w) during cross-linking of BSA, was found to be advantageous as it provides significant stealth effect to the microspheres. Addition of very low amounts of PEG does not contribute to this property, and higher concentration (>50 %) interfere with cross-linking of BSA matrix. This could compromise the integrity of microspheres, resulting in shorter life span and works against sustained drug delivery. Embedding of PEG at 37.5% can produce a partial stealth effect; however, the effect lasts the full life of the microsphere. This is because unlike surface cross-linking, where only surface BSA is cross-linked (once the surface is breached, entire content of microsphere is dumped resulting in burst release), the entire matrix is cross-linked and embedding of PEG during cross-linking helps in providing continual stealth effect even when the microsphere surface erodes over time. This simple embedding technology could enhance the delivery of variety of drugs and lengthens continual delivery with longer retention time in systemic circulation. In contrast, other methods of covalently linking PEG to the drug molecules results in new a moiety, with a changed chemical structure and properties. The resultant is a rather large molecule that could have a pronounced stealth effect, but very likely with obscured target binding sites and may not produce desired therapeutic outcomes. Therefore the PEGylated drug conjugate must cleave in order for drug to bind to the target or to enter the cell resulting in a desired pharmacological effect, which may not be feasible in many instances.

5. Conclusion

The non-covalent and easy PEG embedding technology in non-toxic, cross-linked BSA microspheres, described in this study is free from aforementioned defects and the drug will remain in the original chemical structure in the resulting microspheres. The observed stealth effect to the microspheres could prolong their systemic circulation for improved targeting and enhanced efficacy. This technology can be used to reduce the dosage schedule, which could result in better patient compliance and potentially be cost effective. Further studies are required to improve this technique for even more compact and smaller microspheres with entrapped PEG to provide superior stealth effect, resulting in much longer systemic circulation time and is a subject for future communications.

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