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Hemolysate-filled polyethyleneimine and polyurea microcapsules as potential red blood cell substitutes: effect of aqueous monomer type on properties of the prepared microcapsules

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Abstract

In this paper, we describe the synthesis and characterization of rabbit hemolysate-filled polyethyleneimine (PEI)- or polyurea (PU)-type artificial red blood cells (ARBCs) with different membrane compositions. These microcapsules were prepared by making use of the interfacial polymerization (IP) reaction between the water-soluble amine monomers (triethylamine (TEA), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGATA), diethylenetriamine (DETA), tetramethyl diaminomethane (TMDAM), piperazine hexahydrate (PPHH), L-lysine monohydrochloride (LLMH) or PEI) and 2,4-toluylene diisocyanate (TDI) as an oil-soluble shell monomer. The resultant microcapsules were spherical and with mean diameters of 8.71–63.33 μm. Microcapsules having sulfonic acid groups on their surfaces were prepared by using a combination of the functional amines (DETA, LLMH or PEI) and 4,4′-diaminostilbene-2,2′-disulfonic acid (DASSA). Oxygen-binding abilities of the ARBCs were measured by a Clark-type oxygen electrode. The obtained results revealed that the highest oxygen-binding abilities were obtained with the PU-ARBCs prepared with DETA alone or in combination with EGATA. Unfortunately, these microcapsules exhibited large diameters and wider size distribution curves (span values (S) = 1.3, 1.7, geometric standard deviation (σ_g) = 1.85, 2.18, respectively). However, the novel ARBCs (sulfonated PU-PEI graft copolymer membrane microcapsules (SPU/PEI-ARBCs)) prepared had good oxygen affinity, the smallest mean diameter (d = 8.71 μm) and the best distribution (S = 0.9, σ_g = 1.48) and a flow behavior identical to rabbit RBCs. Therefore, these unique microcapsules can be recommended for scale-up considerations as a promising blood substitute.
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Keywords: Polyurea microcapsules; Interfacial polymerization; Hemoglobin microencapsulation; Polyethyleneimine membrane microcapsules

1. Introduction

In recent years, the demand for blood substitutes for transfusion has become increasingly important. This is because the supply of blood is dependent on blood donation from volunteers at present and chronic shortage of blood for transfusion has become a serious problem. In addition, it is necessary to match blood groups at the time of transfusion, and care is necessary to avoid infection of recipients with viral diseases, such as serum hepatitis. Moreover, HIV donor blood for transfusion may be one of the causes of many immunologically infectious diseases, e.g. AIDS (Muramatsu et al., 1982; Kato and Tanaka, 1985; Chang, 1998).

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In view of this, the development of microcapsule-type artificial red blood cells (ARBCs) with the aim of using them as a substitute for natural ones in transfusion has been attempted so far using collodion (Chang, 1972, 1997). However, the membranes made of collodion, polystyrene or silicon rubber failed to be biodegradable (Lévy et al., 1982). Recently, microencapsulated hemoglobin (HB) with lipid membranes or biodegradable nanocapsules are being developed as blood substitutes (Kato and Tanaka, 1985; Chang, 1997); but, conventional liposomes hardly retain stably aqueous globular proteins, such as HB.

One of the most promising immobilization process is the interfacial polymerization (IP) or polycondensation technique, where the condensation of the polymer at the oil/water interface is initiated in the presence of an oil-soluble monomer and an ultrathin synthetic polymer film is thus formed around each microdroplet. Successful attempts have been made to prepare polyamide (PA)-type ARBCs containing hemolystate by making use of the adopted technique (Arakaw and Kondo, 1980; Muramatsu and Kondo, 1980). However, the formation of strong, stable PA membrane requires rigorous conditions, such as high concentrations of a strong diamine base (≈0.45 M) and a dichloride dispersed in a polar solvent mixture (Chang, 1972; Poncelet et al., 1994). Recently, sheep RBCs-loaded polyurea (PU) microcapsules of a mean diameter of less than 10 μm were prepared under mild conditions by using a biocompatible IP procedure (Kondo et al., 1996). In fact, there are still lots of variables (e.g. the type of water-soluble monomers) that could be expected to play an important role in their oxygen affinity before putting them into clinical trials as ARBCs.

The IP technique was also proposed involving the formation of a polyethyleneimine (PEI) membrane crosslinked by an acid dichloride under mild conditions of low pH, ionic strength and osmotic pressure, which are better suited for the encapsulation of cells and enzymes than those using PA membranes (Povey et al., 1986, 1987a,b; Poncelet et al., 1994). But a membrane made of nylon failed to be biodegradable and showed high affinity to platelet adhesion (Lévy et al., 1982; Muramatsu et al., 1982). Additionally, the reactive nature of the membrane formation process due to the release of acid chloride during the PA crosslinking reactions (Poncelet et al., 1994), impose a limit on the extent to which such conditions are suitable for HB manipulation.

Hence, we prepared in this work PEI membrane-bound microcapsules, as well as sulfonated PU-PEI graft copolymer membrane microcapsules containing HB (SPU/PEI-ARBCs) by using the IP reaction between the PEI or (PEI/4,4′-diaminostilbene-2,2′-disulfonic acid (DASSA)) monomers mixture and 2,4-toluylene diisocyanate (TDI). The properties of these microcapsules (such as oxygen-binding ability, the most important function of any RBC substitute, leakage of HB and size and size distribution) were investigated and compared with those of pure PU membranes (prepared without PEI). The effect of addition of DASSA to the aqueous emulsion phase, as well as variations in the type and combination of monomers on the characteristics of the prepared microcapsules were also studied.

2. Experimental procedures

2.1. Materials

Bovine serum albumin (BSA) was supplied by Nutritional Biochemicals Corp., USA; ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGATA) and DASSA were obtained from Wako Pure Chemicals (Tokyo), triethylamine (TEA), diethylenetriamine (DETA), tetramethyl diaminomethane (TMDAM), piperazine hexahydrate (PPHH), L-lysine monohydrochloride (LLMH), PEI (50% in water), TDI, Tween 20 and Span 85 were purchased from Sigma Chemical Co. (St Louis, MO, USA). All reagents were used as received. The other chemicals, such as cyclohexane and chloroform, were of analytical reagent grade.

2.2. Methods

2.2.1. Preparation of the rabbit hemolysate

The hemolysate used was prepared by a hypotonic hemolysis of washed rabbit RBCs obtained by centrifugation of rabbit whole blood at 3000 rpm (Spectra Centrifuge, UK; Model: Merlin 506) to remove plasma and particulates other than RBCs (Muramatsu et al., 1982).

2.2.2. Preparation of the ARBCs

The IP technique described by Chang (1972) was redesigned to encapsulate rabbit hemolysate in the following way: Firstly, 10 ml of the aqueous solution (5 ml of hemolysate solution), 5 ml of 0.125 M sodium carbonate solution containing 1% (w/v) albumin and, unless otherwise specified, a predetermined concentration (0.2 M) of a functional amine (as the shell-forming water-soluble monomer) was mechanically emulsified in 50 ml of the continuous organic phase (cyclohexane containing 15% (v/v) Span 85 as an emulsifier) by stirring with a mechanical stirrer (Wheaton Instruments, Millville, NJ, USA) at 1500 rpm for 1.5 min at room temperature to yield a water-in-oil emulsion. Subsequently, without stopping the stirring, 50 ml of the oil-soluble shell-forming monomer solution (0.115 M TDI in chloroform) was quickly added into the emulsion vessel to initiate the IP reaction between the amine and TDI at the water/oil interface, and the aqueous droplets were encapsulated to form microcapsules. At the end of the reaction period (2.5 min), a further 50 ml of cyclohexane was added into the reactor vessel to dilute the dispersion and stop the IP reaction. Then, the newly formed PU-ARBCs were collected by centrifugation and transferred immediately into water with the aid of Tween 20 (50% (v/v)), a non-ionic dispersing agent. The ARBCs dispersion, thus obtained was centrifuged and the separated microcapsules were washed repeatedly with deionized water to remove traces of organic solvent and excess surfactant. After manufacture, the resultant suspension obtained was then dialyzed against an isotonic phosphate buffer solution (PBS, pH 7.4), dispersed finally in the same medium and stored in a refrigerator at 4 °C until analysis. Leakage of HB was detected from all of the freshly prepared microcapsules and those stored over a period of 6 months (Table 1). All the batches were triplicated.

Various amines (TEA, EGATA, DETA, TMDAM, PPHH and LLMH) were used separately in the aqueous emulsion phase for the preparation of the pure PU membrane component (Table 1). On the other hand, crosslinked PEI membrane-based microcapsules containing hemolystate (PEI-ARBCS) were formed, under the optimum conditions described above, by a polycondensation reaction between the PEI reactant (at varying concentrations: 5 and 7.5% (w/v)) in the buffered aqueous emulsion phase and 0.115 M of the complementary monomer (TDI) in the organic solvent phase. Sulfonated PU-PEI graft copolymer membrane microcapsules (SPU/PEI-ARBCs) were similarly

Table 1
Effect of aqueous monomers type and their combinations on characteristics of the prepared ARBCs

Aqueous monomer	Concentration	pH of the aqueous phase	Code	Size distribution parameters					Leakage of HB from microcapsules	
				d (μm)	d_{vs} (μm)	d_g (μm)	σ_g	S	After preparation	On storage
TEA	0.20 M	11.27	I	14.39	12.0	14.13	1.78	1.2	–	–
EGATA[a]	0.10 M	7.4	II	20.74	15.94	20.89	2.12	1.5	–	++
EGATA[b] + DETA	0.065 M + 0.135 M	9.48	III	11.02	8.03	10.47	2.18	1.7	–	–
TMDAM	0.20 M	10.90	IV	63.33	52.29	70.8	2.18	1.5	+++	+++
PPHH[a]	0.10 M	10.81	V	9.26	7.98	8.91	1.66	1.10	–	–
LLMH[a]	0.10 M	9.25	VI	26.35	23.33	26.92	1.68	1.15	+++	+++
LLMH + DASSA	0.135 M + 0.065 M	7.65	VII	10.66	9.81	10.72	1.53	0.80	–	+
DETA	0.20 M	10.93	VIII	14.89	12.13	14.13	1.85	1.3	–	–
DETA + DASSA	0.135 M + 0.065 M	10.2	IX	13.64	10.71	12.59	1.88	1.5	–	–
PEI	5% (w/v)	10.7	X	12.24	9.85	11.48	1.87	1.4	–	–
PEI	7.5% (w/v)	10.75	XI	12.26	9.86	11.74	1.82	1.4	–	–
PEI + DASSA	5% (w/v) + 0.065 M	9.92	XII	8.71	7.79	8.32	1.48	0.9	–	–

TEA: triethylamine; EGATA: ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DETA: diethylenetriamine; TMDAM: tetramethyl diaminomethane; PPHH: piperazine hexahydrate; LLMH: L-lysine monohydrochloride; DASSA: 4,4′-diaminostilbene-2,2′-disulfonic acid; PEI: polyethyleneimine. d: average mean diameter (μm), d_{vs}: volume–surface mean diameter (μm), d_g: geometric mean diameter (μm), σ_g: geometric standard deviation, S: span value ($=d_{0.9} - d_{0.1}/d_{0.5}$).

[a] Microcapsules were not formed at 0.2 M concentration of the aqueous monomer.

[b] Microcapsules were not formed with EGATA/DASSA mixtures.

prepared through the IP process using a mixture of core polymer (5% (w/v) PEI) and DASSA (0.065 M) in the aqueous emulsion phase and TDI at 0.115 M concentration in the organic phase.

In another set of experiments, a combination of monomers was also used for preparing PU microcapsules through the complete process by using the following initial conditions in the aqueous emulsion phase: (a) a mixture of DETA and EGATA at a molar ratio of 1:0.482; (b) a mixture of DETA (or LLMH) and DASSA at a molar ratio of 1:0.482. The IP reaction was carried out by using a constant total monomers concentration (0.2 M) in the aqueous emulsion phase and a fixed TDI concentration (0.115 M) in the organic phase (Table 1).

Optical observation of the final microcapsules was carried out with the aid of an Axiolab optical microscope (Carl Zeiss, Germany), and the selected samples were stained (Giemsa stain, Coles, 1986) and photomicrographed by means of an automatic camera (Carl Zeiss) attached to the microscope (Fig. 2). The visible electronic spectrum of the oxygenated capsules suspension in PBS solution (pH 7.4) and native HB were measured by a double-beam spectrophotometer (Unicam SP 1750 UV Spectrophotometer, Pye Unicam Ltd, Cambridge, UK) at 540 and 578 nm using the Opal glass method (quartz cuvettes combined with Opal glass), and the same medium was used as a blank.

2.2.3. Determination of size and size distribution of the ARBCs

The size of microcapsules was measured by a Coulter Multisizer interfaced with a personal computer (Coulter Electronics Limited, Northwell Drive Luton, Bedfordshire, UK) after appropriate dilution with Isoton II electrolyte solution The distribution was expressed by the fraction, f_i, of the microcapsules with the same diameter, d_i. A plot of f_i versus d_i gave the size distribution curve (Fig. 3). The particle diameters (d_{16}, d_{50}, d_{84}) were calculated using the distribution data presented on a log-normal probability paper, and thus the geometric mean particle diameter (d_g) was given by d_{50} and the geometric standard deviation (σ_g), a representation of size distribution, was given by $(d_{84}/d_{16})^{1/2}$ (Poncelet De Smet et al., 1989). The size dispersion was also estimated through the span (S), which defined the centered diameter range containing 80% of the microcapsules (microcapsules in number distribution) divided by the mean diameter. The mean diameter (d) of the microcapsules was the medium diameter at which a vertical line divides the area under the distribution curve into equal parts. The reported values are means of two or three sample results.

2.2.4. Evaluation of oxygen-binding ability of the ARBCs

Measurement of oxygen concentration was carried out by a procedure based upon those reported previously (Kato and Tanaka, 1985; Kondo et al., 1996) with certain modifications. Generally, the oxygen-binding ability of the ARBCs suspension in the PBS solution (pH 7.4) was measured in a closed thermostatically controlled (37 ± 0.5 °C) chamber having two openings and attached to a Clark-type oxygen electrode (Oxygen Monitor Apparatus, Yellow Springs Instrument Co., Inc., Yellow Springs, OH, USA). A suspension of the ARBCs at 10% (v/v) particle concentration and the PBS solution (pH 7.4), each saturated with air at 25 °C by means of airing, were used as samples for measurements. The air-saturated PBS solution was also used as the standard solution having a constant amount of oxygen (230 nmol/ml) at room temperature (Umbreit et al., 1964). A 4 ml of the cells suspension (or the PBS solution) was added into the chamber through a vent in the chamber stopper and allowed to stir at 50 rpm and 37 ± 0.5 °C. A nitrogen gas was introduced into the microcapsules suspension with stirring to replace oxygen in the cell contents. Subsequently, a 2 ml of the air-saturated PBS solution at 37 °C was introduced into the chamber solution to give a suspension having a given saturation of oxygen, and then the amount of oxygen absorbed by HB in the ARBCs was calculated by subtracting the amount of residual oxygen in the suspension (after a steady resting conditions) from the oxygen content of the reference sample (PBS solution). Duplicate microcapsule batches generally showed good reproducibility in binding properties.

2.2.5. Measurement of viscosity of the ARBCs suspensions

The flow properties of suspensions (16 ml)of the ARBC were measured by a Brookfield Digital viscometer (Model DV-II+ version 3.0, Brookfield Engineering Lab., Inc., Stoughton, MA, USA). The temperature was maintained at 30 ± 0.5 °C throughout

the measurement ($n = 3$). Measurements were also carried out on rabbit erythrocyte suspensions prepared as previously reported (Muramatsu et al., 1982). The slope of linear portion of flow curve for a suspension gives its apparent viscosity and the relative viscosity was obtained as the ratio of the apparent viscosity of suspension to that of the suspending medium.

2.2.6. Statistical analysis

Data on the oxygen-binding profiles of the ARBCs were expressed as the mean ± S.D. ($n = 3$) by using the Prism-3 GraphPad computer program. Data were also analyzed for ANOVA with Bonferroni's post-test for multiple comparisons with confidence intervals at 95% as appropriate for oxygen absorption.

3. Results and discussion

3.1. Formation of the ARBCs

Pure PU membrane microcapsules were prepared by the IP process using 15% of Span 85 as an emulsifier, TDI as the oil-soluble monomer, and amines of widely differing chemical structure as the aqueous monomers. The high emulsifier concentration used in the system has two functions: first, to increase the interfacial viscosity and significantly reduce interfacial tension between water and oil phases so that smaller microcapsules may be obtained; second, to adsorb on the water/oil interface to form a layer around the water droplets, thus significantly reducing the rate of droplet coalescence due to the formed steric repulsion forces (Yan et al., 1993; Alexandridou and Kiparissides, 1994).

A shorter emulsifying time of 1.5 min was recommended in all the experiments in order to avoid chemical modification of HB by the crosslinker (Lévy et al., 1982; Povey et al., 1986). PU membranes formed using the various water-soluble monomers are generally strong, stable and spherical in shape.

Sulfonated microcapsule surfaces were prepared by making use of the polycondensation reaction between TDI and the mixture of the water-soluble amine with DASSA. Primary experiments revealed that 0.2 M of the aqueous amines mixture (whose molar ratios of DASSA to the other amine was 1:2.077) is the total concentration of choice for the selected monomers. The presumptive chemical reaction of PU membrane formation is depicted in Fig. 1.

It is well known that the shell formation of microcapsules by the IP reaction proceeds as follows (Kondo, 1991): (1) initial stage of polymerization, (2) formation of the first incremental layer of polymer surrounding the drops and (3) further growth of the shell for the microcapsule. The second step is the process wherein the chain of prepolymer molecules precipitates at the interface, leading to the formation of the first incremental layer. In fact, pure PU and nylon membranes are formed by the polymerization reaction between a diamine and a diisocyanate or dichloride, respectively, forming mostly linear chains, most likely interlaced to form a net (Poncelet et al., 1994; Kondo et al., 1996).

Formation of the pure polymer membranes during the IP process is generally considered to occur via the passage of a diamine from the aqueous into the organic phase so that for aqueous droplets the membrane forms outward. This contributed to the formation of most condensation polymers in the organic phase. However, rapid membrane formation at the interface progressively impedes transfer of further diamine into the organic side of the interface so that subsequent thickening and growth of the membrane surrounding the droplets occurs more slowly in the organic phase. This transfer rate is also reduced when the polarity of an organic solvent, the diffusivity coefficient of amines in oil phase and the pH of the diamine solution are decreased (Morgan and Kwolek, 1959; Poncelet et al., 1990; Zhang et al., 1995).

In the current study, spherical and rigid PEI membrane-based microcapsules (PEI-ARBCs) and sulfonated PU-PEI graft copolymer membrane microcapsules containing hemolysate (SPU/PEI-ARBCs) were reproducibly prepared, respectively, by the crosslinking of the polycationic electrolyte, PEI polymer (or PEI/DASSA mixtures) by TDI in the polycondensation reactions (Fig. 2). PEI ($(CH_2CH_2NH)_n$) includes a large spectrum of water-soluble polyamines of variable molecular weight with varying degrees of modifications. All PEIs produced by the ring-opening cationic polymerization of ethyleneimine are believed to be highly branched, containing primary, secondary and tertiary amine groups in the ratio of approximately 1:2:1 (Horn, 1980). Crosslinking of PEI thus forms a three-dimensional network, which increases the

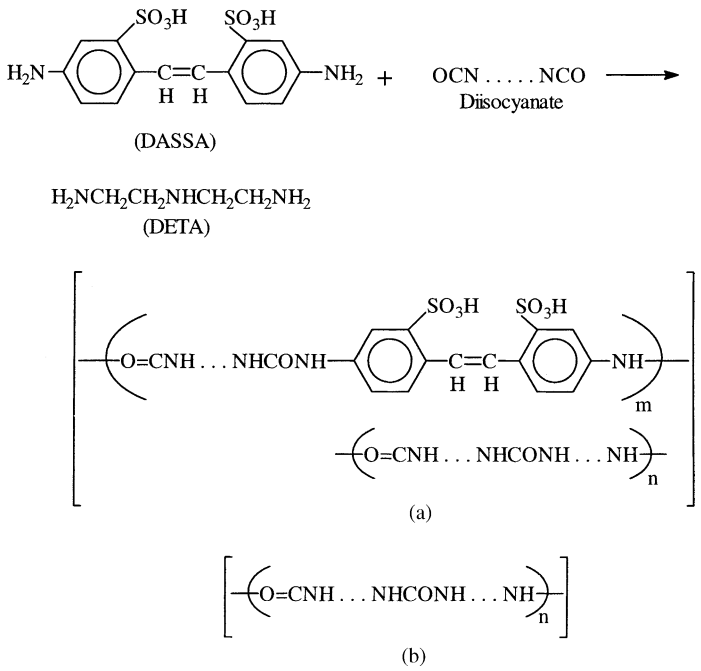

Fig. 1. Structural formulae of (a) sulfonated and (b) nonsulfonated representative microcapsule membrane component.

rigidity of the PEI membrane (Poncelet et al., 1994). Seki and Okahata (1986) stated that crosslinking between PEI and the polyamide (PA) chains had yielded an extended network for which zwitterionic polarization between the amine and carboxylate components could have substantial effects on the functional properties of the semipermeable membrane.

PEI membrane formation is slightly different from that of pure PU. Firstly, PEI is a polar compound, insoluble in organic solvents (Horn, 1980). As the PEI does not enter the organic phase, then it is postulated that the crosslinker TDI is conveyed into the aqueous phase (i.e. a PEI layer which forms near the organic/aqueous interface), presumably due to the additional surfactant from albumin present in the aqueous phase. Thus, the reaction would tend extensively towards the aqueous side of the organic/aqueous interface due to the hydrophilic properties of crosslinked PEI. Povey et al. (1987a) reported that during PA/PEI membrane formation, no PEI was present in the organic phase, indicating that the microcapsule membrane had formed inwards contrary to the membranes formation of pure PA systems. Accordingly, the pH in the aqueous phase and the polarity of the organic solvent, should not affect the reaction kinetics or PEI membrane characteristics to the same extent as was the case with other membrane systems. In addition, the ionic strength and osmotic pressure in the microcapsule core can be greatly lowered, permitting the encapsulation of sensitive biological cells (Poncelet et al., 1994). The preparation of stable PEI microcapsules without a diamine in the aqueous phase is a direct evidence indicating chemical incorporation of the PEI into the membrane during formation, and not merely physical inclusion (Poncelet et al., 1994).

3.2. Characterization of the prepared ARBCs

3.2.1. Micrometric properties

According to the liquid–liquid agitation theory, the dispersion produced by agitating two immiscible liquids is determined by the breakdown and the coalescence of the droplets (Chatzi et al., 1991; Alexandridou and Kiparissides, 1994). After an initial transient time period, a dynamic equilibrium between these two phenomena is established. Variations in aqueous emulsion phase conditions may affect the final droplet size distribution, probably by changing

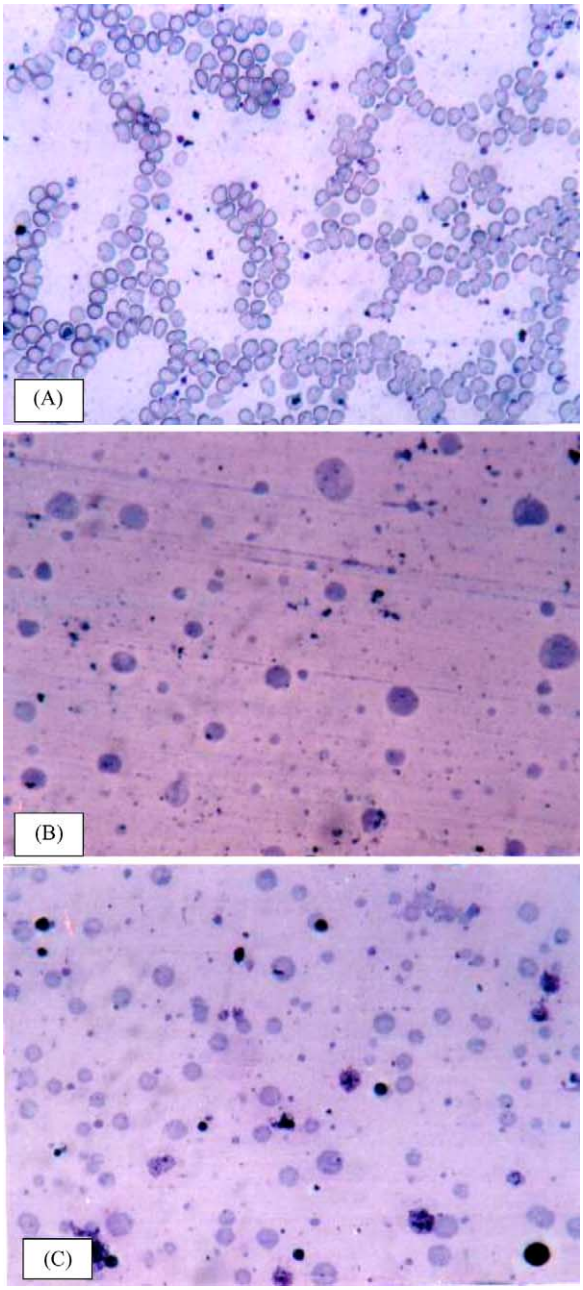

Fig. 2. Photomicrographs of (A) rabbit RBCs, (B) PEI-ARBCs (code XI) and (C) SPU/PEI-ARBCs (code XII) (Giemsa stain, 10×40).

the mechanisms of droplet breakage and coalescence (Alexandridou and Kiparissides, 1994).

One of the most common particle size distribution factors which may provide good information about the possible symmetry of the distribution is the log-normal probability function, expressed by the following equation (Poncelet De Smet et al., 1989):

$$\mathrm{d}(P) = \frac{\mathrm{d}(\log d)}{\sqrt{2\pi/100}(\log \sigma_\mathrm{g})} \times \left(\exp\left[-\left(\frac{\log d - \log d_\mathrm{g}}{\log \sigma_\mathrm{g}} \right)^2 \right] \right)$$

where P is the percentage of microcapsules having a diameter smaller than the average mean diameter of the microcapsules (d). The log-normal particle size distribution is then characterized by two parameters: d_g, the geometric mean particle diameter; and σ_g, the geometric standard deviation.

The distribution parameters of the microcapsules shown in Table 1, indicate that the d_g values calculated from the log-normal distribution function are in good correlation with the average mean diameters. The PU microcapsules had mean diameters of between 8.71 and 26.35 μm, except for TMDAM microcapsules (code IV) which had the largest microcapsule size ($d = 63.33$ μm) (Table 1). The particle size distribution of the systems exhibited a unimodal form (Fig. 3), indicating that erosive droplet breakage becomes less important (Chatzi et al., 1991). Thus, the size of the emulsion aqueous droplets before membrane formation would seem to be of more importance in determining final microcapsule size than processes occurring after the start of membrane formation. Mikami (1994) stated that the electrical repulsive force by the surfactant contribute to form a stable emulsion and prohibit droplets coalescence.

The size distribution curves of the microcapsules (Fig. 3, codes I–IV, VI, VIII–XI) were found to be wider and with lower frequency percent. In addition, the span (S) values (which represent the width of distribution) and the values of σ_g (which represent the symmetry of distribution) appeared to be higher for these microcapsules (Table 1). Therefore, these systems are not recommended. On the contrary, a size distribution which is narrower, shifted towards the smaller microcapsule range and nearly symmetrical around the modal diameter was obtained on using 0.1 M PPHH (Fig. 3, code V). The microcapsules distribution obtained had lower S and σ_g (values = 1.10 and 1.66, respectively). Also, as shown in Table 1,

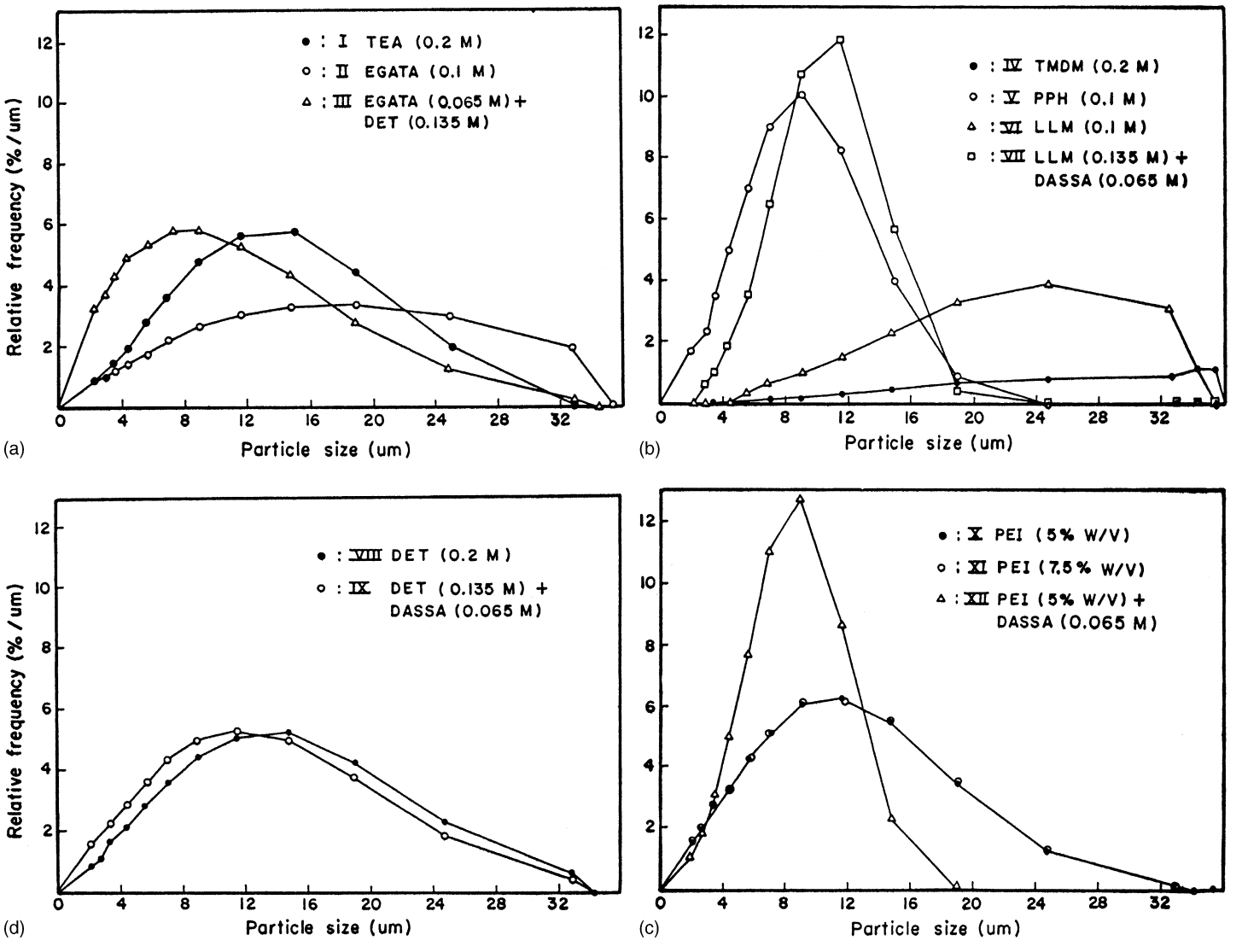

Fig. 3. Effect of aqueous monomers type and their combinations on particle size distribution of the prepared ARBCs.

they exhibited a smaller arithmetic mean particle diameter ($d = 9.26\,\mu m$) in comparison with those prepared with other diamines (TMDAM (code IV), $d = 63.33\,\mu m$; LLMH (code VI), $d = 26.35\,\mu m$).

The results shown in Table 1 revealed also that using a combination of DASSA (0.065 M) and LLMH (code VII) or PEI (code XII) in the emulsion aqueous phase was associated with a pronounced decrease in particle size, σ_g and S values of microcapsules as compared with those prepared without DASSA (code VI (LLMH), and code X (5% PEI), Table 1). The preliminary studies indicated that there is a certain concentration of DASSA (0.065 M) where an optimum microcapsule size can be attained. It thus appears that using LLMH/DASSA mixtures at a molar ratio of 1:0.482 (code VII; pH of the aqueous phase = 7.65) resulted in a significant reduction in the mean diameter, σ_g and S values by about 60, 14.3 and 27.3%, respectively. On the other hand, SPU/PEI membrane microcapsules (prepared with DASSA/PEI combination, code XII) had the smallest particle size ($d = 8.71\,\mu m$) amongst the tested formulations (Table 1). Poncelet et al. (1994) reported that the pH of the PEI aqueous phase affects the size distribution of PEI microcapsules crosslinked by a dichloride. PEI is a positively charged polymer, the charge increases by protonation of the amine groups with lowered pH. The increased charge on the polymer results in a change in the solution viscosity, affecting the size of droplets formed during the emulsification step. Obviously, addition of DASSA to the PEI aqueous phase led to a decrease in the mean diameter of such microcapsules of up to 3.5 μm, as well as a significant reduction in the σ_g and S values by about 19.8 and 35.7%, respectively (Fig. 3). Each batch of these microcapsules (code XII) contained different populations with high frequency percent of smaller microcapsules (<8 μm) (Fig. 3).

The size distribution of microcapsules prepared with DASSA (codes VII and XII) appeared to be narrower, steeper and nearly symmetric around the modal diameter which was found to offset actually the median diameter, the corresponding relative frequencies (at the modal diameter) increased strongly with disappearance of the right-side bias (Fig. 3). Thus, it seems likely that presence of the acidic monomer (DASSA) in the aqueous phase of the primary emulsion may have altered the interfacial tension between the two liquid phases. This, in turn, affected the average droplet size of the formed emulsion by decreasing the coalescence rate of smaller emulsion droplets with each other and with the embryonic microcapsules during the initial stages of microencapsulation, resulting in the formation of smaller microcapsules.

Generally, it can be seen from Table 1 and Fig. 3 that increasing the PEI concentration from 5% (code X) to 7.5% (code XI) did not affect the average size parameters and size distribution of PEI microcapsules ($d \approx 12.25\,\mu m$, $\sigma_g = 1.82$–1.87, $S = 1.4$). This finding can be interpreted in light of the fact that PEI cannot be partitioned into the oil phase, while TDI has to diffuse on to an oil/water interface to react with PEI to form an initial membrane. Therefore, the strength of the initially formed membranes on an oil/water interface is dependent mainly on diffusivity coefficient and concentration of the TDI in oil phase and independent on PEI concentration. In addition, the formed membranes might be strong enough to withstand the shear stress in the agitator and prevent the droplets from coalescing, irrespective of PEI concentration. Consequently, the microparticle size and distribution will remain unchanged (Chatzi et al., 1991).

Table 1 and Fig. 3 also illustrate that using a mixture of DETA and EGATA (at a molar ratio of 1:0.482, code III) was accompanied by a drastic reduction in particle size ($d = 11.02\,\mu m$) in comparison with EGATA alone (code II, $d = 20.74\,\mu m$). This finding can be explained on the basis that the rapid formation of the interfacial PU with an adequate film thickness (needed to prevent the coalescence of droplets) is believed to be mainly due to the higher reactivity of this polyamine (DETA) with diisocyanates (Mikami, 1994; Mahabadi et al., 1996). Additionally, the effect of DETA monomer (present at a higher concentration (0.135 M) than that of EGATA (0.065 M)) on elevating pH of the aqueous phase (Table 1) could substantially reduce the transfer rate of the acidic monomer (EGATA) into the organic phase, leading to incomplete polycondensation of this monomer, so that the reaction of EGATA and TDI can be greatly minimized. It is also possible that the addition of DETA reduces the surface tension of the primary emulsion aqueous phase and thus reduces the equilibrium particle size due to both a decrease of resistance to droplet breakage and an increase of resistance to coalescence (Chatzi et al., 1991; Mahabadi et al., 1996). The formation of

relatively smaller microcapsules ($d = 14.89\,\mu m$) on using DETA alone (code VIII) in the aqueous phase supports the above suggestions (Table 1 and Fig. 3). However, the σ_g and S values of the DETA/EGATA microcapsules (code III) seemed to be of the greatest values (2.18 and 1.7, respectively; Table 1) and their frequency distribution curve was broader and with low frequency percents (Fig. 3).

3.2.2. Oxygen-binding ability

HB which is composed of iron(II)–porphyrin complexes (hemes) and globin proteins, serves to transport and store molecular oxygen in a living body. Globin protein protects the complex from irreversible oxidation by embedding it separately in the macromolecule. However, the properties of the protein are largely altered when the invariant residues of the amino acid sequences of HB chains are replaced, as in abnormal HB (Antonini and Brunori, 1971, 1975).

Optimization of oxygen-binding properties will invariably involve the microcapsule membrane structure since oxygen must pass through the membrane to reach the core target material. Such transport will depend not only on the membrane chemical nature, thickness and porosity, but also the properties of the encapsulated HB (e.g. its molecular weight, chemical nature and aqueous stability) may be encountered in the oxygen-binding process. Therefore, we have studied the effects of different aqueous monomers on the properties and effectiveness of these microcapsules in binding oxygen (Table 1 and Fig. 4).

Fig. 4 shows the plot of oxygen absorption of various systems against the type of water-soluble monomer used. The results revealed that the immobilized HB entrapped in PU microcapsules prepared with EGATA (code II), TMDAM (code IV), LLMH (code VI) or a mixture of LLMH and DASSA at a molar ratio of 1:0.482 (code VII) exhibited the

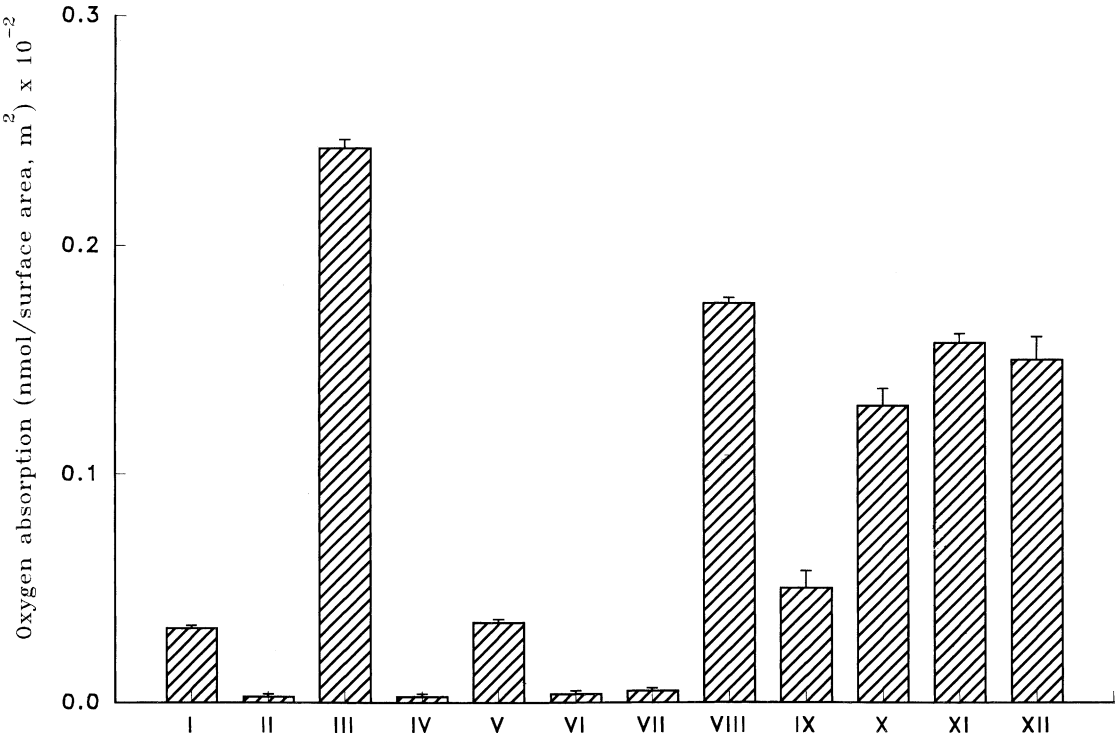

Fig. 4. A histogram showing the effect of aqueous monomers types and their combinations on oxygen absorption of the prepared ARBCs. I = TEA (0.2 M), II = EGATA (0.1 M), III = EGATA (0.065 M) + DETA (0.135 M), IV = TMDAM (0.2 M), V = PPHH (0.1 M), VI = LLMH (0.1 M), VII = LLMH (0.135 M) + DASSA (0.065 M), VIII = DETA (0.2 M), IX = DETA (0.135 M) + DASSA (0.65 M), X = PEI (5% (w/v)), XI = PEI (7.5% (w/v)), XII = PEI (5% (w/v)) + DASSA (0.065 M).

lowest rates of oxygen absorption as compared to other systems. In general, the absorption spectrum of these oxygenated capsules was strongly altered (Fig. 5, codes II, IV, VI, VII) and the characteristic peaks were less definite and broad as compared with those of native oxygenated HB, appearing at 577 and 541 nm (Antonini and Brunori, 1975). A greater leakage of HB was detected from the microcapsule

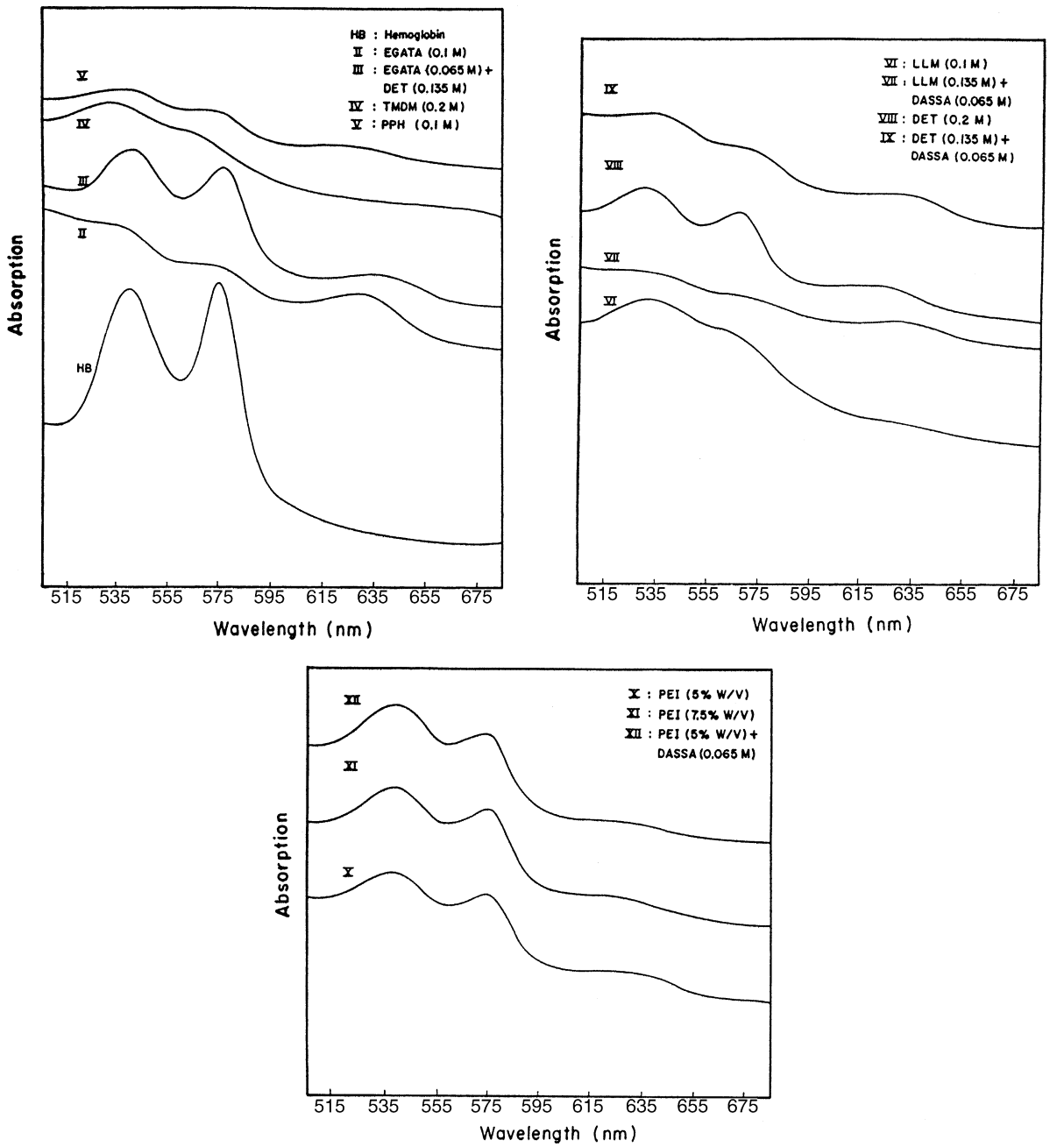

Fig. 5. Effect of aqueous monomers type and their combinations on the visible absorption spectra of the prepared ARBCs.

batches of TMDAM (code IV) and LLMH (code VI) after preparation and on storage (Table 1). This observation suggests that the porosity of microcapsules may be too large for HB release, probably due to the formation of an interfacial PU film which does not have a homogeneous network. This leads to a significant reduction in HB content of the cells and in turn, the low rate of oxygen absorption (Fig. 4). It is also possible that the high pH and ionic strength of the aqueous phase due to the higher monomers concentration used (e.g. code IV (TMDAM 0.2 M), pH 10.9) may promote dissociation of HB (tetramer) into subunits of lower molecular weight, an effect which facilitates passage of HB subunits through the capsular membrane and reduces the HB molecule's affinity for oxygen (Antonini and Brunori, 1971, 1975). On the other hand, the microcapsules prepared with the acidic monomers (EGATA (code II) and LLMH/DASSA mixture (code VII)) at an aqueous emulsion phase pH of ≈7.5 showed no diffusion of HB into water after preparation. On storage, the supernate was slightly colored, indicative of some HB leak (Table 1), but diffusion of HB out of the wall was much slower as compared to TMDAM or LLMH microcapsules. Thus, the nearly abolished oxygen absorptivity of such microcapsules is more likely to be due to severe HB modification during encapsulation, as evidenced from the disappearance of the absorption peaks of oxyhemoglobin (oxy-HB) and the appearance of the characteristic peak of acid methemoglobin (HB^+) at 630 nm (Fig. 5) (Antonini and Brunori, 1975). Sacco et al. (1989) reported that the covalent coupling of polyanionic molecules (e.g. carboxylated or sulfated dextran) onto oxy-HB was capable of reducing the oxygen affinity of HB in the same way as the natural effector, 2,3-diphosphoglycerate (2,3-DPG), i.e. by reacting specifically with the amines of the protein allosteric site (phosphate-binding site). Also, the use of lower pH values was reported to be unsuitable for the formation of PU membranes and immobilization of enzymes (Rambourg et al., 1982). Under these conditions, incomplete polycondensation of the monomer, modification of HB by the interaction of globin portion with the crosslinking agent and subsequent incorporation of the HB molecule into the capsular membrane may occur. This would lower the concentration of HB in the capsules, enhance dissociation of HB molecules into subunits and lead to the formation of less-integral membranes, thereby modifying the oxygen absorption curves (Arakaw and Kondo, 1980). Supporting this conclusion is that with magnetic HB microcapsules prepared in a similar manner with terephthaloyl chloride (TPC), their rapid hydrolysis and destruction by trypsin indicated HB to be accessible in the outer membrane layers (Povey et al., 1986).

The oxygen-binding abilities gained by using TEA (code I), PPHH (code V) or DETA/DASSA mixture at a molar ratio of 1:0.482 (code IX) as the water-soluble monomers are shown in Fig. 4. Although HB did not diffuse from all of these microcapsules (Table 1), the oxygen absorption rates of such microcapsules are still low, which might be due more to partial HB combination with the membranes, HB precipitation within the core during storage, or arising from the high pH (10.2–11.27) of the aqueous emulsion phase generated by the monomers used (Antonini and Brunori, 1975; Povey et al., 1986). This can be observed from the absorption spectra of the encapsulated HB which are not identical with that for normal HB, indicating some changes in chemical nature of HB inside the microcapsules (Fig. 5). It is also clear from Fig. 4 (TEA (monoamine), code I; PPHH (diamine), code V; DETA (triamine)/DASSA (diamine) mixture, code IX) that as the number of amino groups in the molecule increased the oxygen affinity of the immobilized HB increased proportionally, irrespective of the concentration of monomer used. Obviously, oxygen affinity was thus significantly increased for DETA/DASSA microcapsules (code IX) when compared with TEA (code I, $P < 0.01$) and PPHH (code V, $P < 0.05$) microcapsules. On the other hand, microcapsules prepared using DETA alone at 0.2 M concentration (code VIII) or a 0.2 M mixture of DETA and EGATA at a molar ratio of 1:0.482 (code III) exhibited the highest oxygen absorption rates amongst the tested formulations ($P < 0.001$, except for VIII versus XI: $P < 0.01$) (Fig. 4). The possible crosslinking of HB by polymers may affect some chemical functions involved in the mechanism of oxygen binding and lead to a bound protein with a high oxygen affinity (Mohr et al., 1980). However, as it is difficult to precisely state the final mean concentrations of the macromolecule in the microcapsule populations, the greater resistance of their membranes to HB diffusion out of the capsules and their unaltered absorption peaks may reflect structural

differences of the formed cells and/or indicate the presence of a high concentration of unchanged HB inside the DETA microcapsules (Table 1 and Figs. 4 and 5). Interaction between the polyamines and TDI may thus yield an extended or branched network that could have substantial effects on the stability and functional properties of the formed microcapsules. Yan et al. (1993) reported that PU microcapsules prepared using DETA as a water-soluble monomer had the best physical stability as compared to ethylenediamine, 1,6-hexamethylenediamine (HMDA), triethylenetetramine, tetraethylenepentamine, lysine or piperazine microcapsules. Similarly, Alexandridou et al. (2001) reported that the homogeneity and the rigidity of the polyterephthalamide microcapsule membranes was favored by an increase of the triamine (DETA) versus diamine (HMDA) ratio in the emulsification step. Unfortunately, the average diameters of the DETA microcapsules (codes III and VIII) are larger ($d = 11.02$ and 14.89 μm, respectively) and their distribution curves are wider (Table 1 and Fig. 3).

The use of PEI allowed the preparation of stable HB microcapsules, the membranes of which were resistant to HB diffusion (Table 1) but permeable to oxygen (Fig. 4, codes X, XI and XII). The electronic spectrum peaks of these oxygenated capsules were definite and nearly identical with that for normal HB (Fig. 5). These findings may be interpreted in light of the fact that incorporation of the highly branched polyamine molecule (PEI) into the membrane produces an interfacial, crosslinked film with a homogeneous network. In fact, such incorporation proved to be essential for the physical stability of the microcapsules (Povey et al., 1987b). Also, Povey et al. (1986) prepared stable nylon microcapsules containing PEI and found that at lower PEI concentration (5 g/l), no PEI was released on sonication of these microcapsules indicating that the encapsulated polymer had been totally incorporated into the membrane. As PEI was used at higher concentrations (50 and 75 g/l) in our work, thus it can be expected that excess PEI is present in the core, which may stabilize HB by ionically crosslinking the negatively charged protein molecules inside the microcapsules (Park et al., 1992).

Although PEI microcapsules prepared at different PEI concentrations (5% PEI, code X; 7.5% PEI, code XI) exhibited the same particle size (≈12.25 μm) (Table 1) and the measurements were conducted on the same number of particles, use of a higher concentration of PEI increased significantly the oxygen affinity of encapsulated HB ($P < 0.001$) as compared with the lower concentration (Fig. 4), further confirming that increased core PEI concentration stabilizes HB in the microcapsule core.

The HB microcapsules (SPU/PEI-ARBCs) prepared with a mixture of 5% PEI and DASSA at 0.065 M concentration (code XII) in the aqueous emulsion phase had good oxygen-binding properties (Fig. 4), unaltered absorption peaks (Fig. 5) and seemed to give the best results regarding the average size ($d \approx 8.71$ μm) and the distribution of diameters (Table 1 and Fig. 3). On the other hand, the oxygen-combining ability of such microcapsules (code XII) was increased significantly ($P < 0.01$) as compared to code X microcapsules (prepared without DASSA), whereas no significant difference was observed between the oxygen-binding profiles of these microcapsules and XI microcapsules ($P > 0.05$) (Fig. 4). Generally, the insignificant differences observed between the oxygen affinity of the freshly prepared PEI microcapsules and those stored over a period of 6 months at 4 °C indicate the beneficial effects of such microcapsules on the stability of the encapsulated HB (data not shown).

3.2.3. Flow properties of the ARBCs suspensions

It is well known that the flow curves of the native erythrocyte suspensions are of a pseudoplastic type and their flow properties are thus well fitted for the Casson plots (shear rate$^{1/2}$ versus shear stress$^{1/2}$) (Kato et al., 1983).

Fig. 6 illustrates the Casson plots for the flow of SPU/PEI-ARBC suspensions (at concentrations of 30, 40 and 50% (v/v) in phosphate buffer, pH 7.4) and rabbit RBCs suspension (40% (v/v)). The plots yield a straight line for all the suspensions. The ARBC suspensions displayed a decreasing viscosity with an increasing shear rate, indicating that the flow type of this suspension was of pseudoplastic as in the case of rabbit RBCs suspension (pH 7.4). These results indicated similarity with those of Arakaw and Kondo (1980) on polyamide microcapsules containing sheep hemolysate. They pointed out that the capsules particle–particle interactions may play a part in the occurrence of this type of flow as they should behave

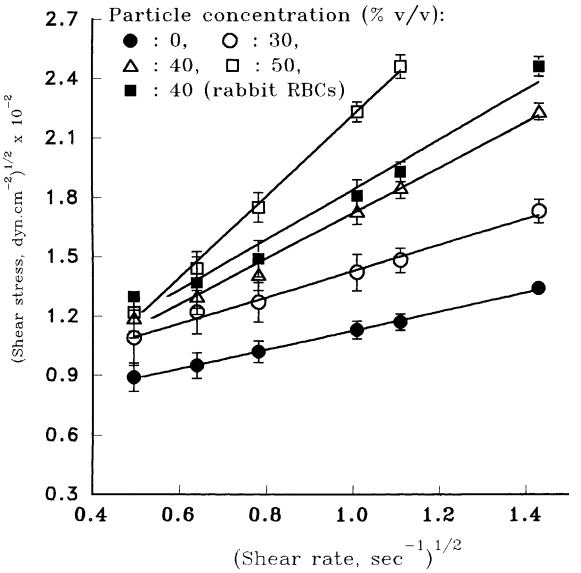

Fig. 6. Casson plots at various particle concentrations of SPU/PEI-ARBCs (code XII) suspensions in phosphate buffer solution (pH 7.4). Each point represents the mean ± S.D. ($n = 3$).

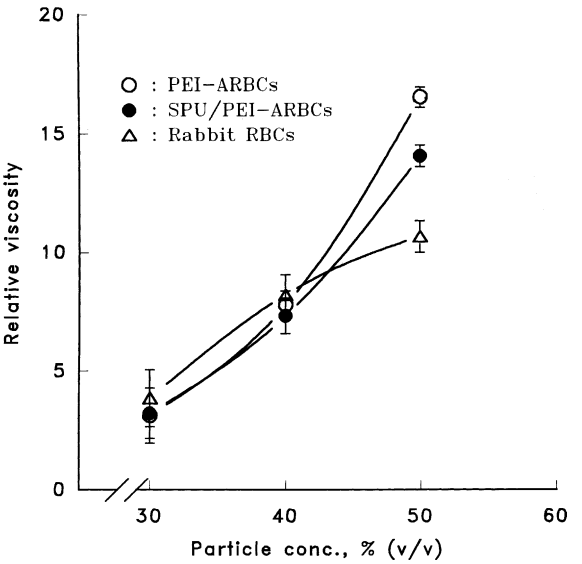

Fig. 7. Relative viscosity of PEI-ARBCs (code XI), SPU/PEI-ARBCs (code XII) and rabbit RBCs suspensions as a function of particle concentration in suspending medium (phosphate buffer, pH 7.4). Each point represents the mean ± S.D. ($n = 3$).

as rigid particles in shear flows in view of their very small size and very high internal pressure.

In Fig. 7 are shown the relative viscosity for the ARBCs and rabbit RBCs suspensions as a function of particle concentration in phosphate buffer solution (pH 7.4). The relative viscosity of the rabbit RBCs suspension is slightly higher than that of the ARBCs suspensions when compared at 30 and 40% particle concentrations and it tends to rise remarkably with increasing particle concentration for all the suspensions. This would be attributed to increase in the total capsule/medium interface area caused by increase in the number of particles in unit volume of suspension, which should increase the resistance to flow of suspension (Arakaw and Kondo, 1980).

Fig. 8 shows the relative viscosity for 40% (v/v) suspensions (pH 7.4) of the SPU/PEI-ARBCs and rabbit RBCs as a function of dextran concentration. It was found that the relative viscosity of the ARBC suspension, just as that of the rabbit RBCs suspension, is reduced with increasing dextran concentration. Similar results were reported on hemolysate-loaded liposome suspensions (Kato et al., 1983). They found that the relative viscosity of the cell suspensions depends dominantly on the dextran concentration in the suspending

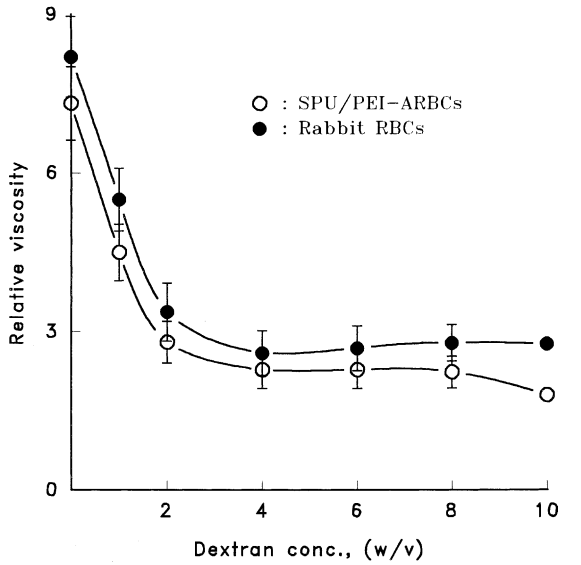

Fig. 8. Relative viscosity of SPU/PEI-ARBCs (code XII) and rabbit RBCs suspensions as a function of dextran concentration in suspending medium (phosphate buffer, pH 7.4). Particle concentration: 40% (v/v). Each point represents the mean ± S.D. ($n = 3$).

medium, which would be due to a weak excluded volume effect caused by the adsorption of dextran on the cells.

4. Conclusion

Variations in the type of water-soluble monomers used in interfacial polymerization have been shown to affect various microcapsule parameters, indicating that amine–TDI interaction plays a major role in the manufacturing process. Although the microcapsules prepared by using DETA alone or DETA/EGATA combination had the highest oxygen affinity, wider distribution curves were obtained. A simpler approach for minimizing microcapsule diameter is to add DASSA monomer to the aqueous phase before the particle formation step, suggesting that DASSA has some beneficial effects on microcapsule properties. The proposed method of encapsulation demonstrated also that crosslinked PEI membrane-based microcapsules can be used for entrapping HB because of their good oxygen affinity and unaltered absorption spectrum peaks. Moreover, the sulfonated form of these microcapsules (SPU/PEI-ARBCs) gave the best results regarding the average size and the distribution of diameters. Therefore, these microcapsules could in principle be used for many other types of applications beyond the scope of this present work.

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