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Synthesis of Thromboresistant Heparinized Polysilicone Using Radiation Grafting

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ABSTRACT

Gamma rays from ^{60}Co were used to graft chloromethylstyrene on samples of polysilicone, followed by quaternization with pyridine, then reaction with sodium heparin to replace chloride ions in the samples with heparin anions. Extraction tests on the heparinized samples with normal saline solution showed a maximum heparin removal rate of 5×10^{-8} g/cm^2/min. The heparinized polysilicone was found to be more blood compatible than untreated polysilicone in a rabbit ex vivo bleeding test.

INTRODUCTION

Much research time has been spent in the attempt to find plastic materials that will not cause clotting (thrombosis) when placed in contact with blood. Several investigators have shown that polymers containing chemically bound heparin, or coated with heparin, exhibit thromboresistance [1-4].

Commercial heparin preparations make up a family of mucopolysaccharides having similar repeating units and functional groups [5]. These substances are not molecularly homogeneous but show considerable variation in molecular size [6], variations in the ratio of glucuronic acid to iduronic acid [7], alterations in the amount of N-sulfation [8], and differing extents of N-acetylation [9]. Sodium heparin consists of positive sodium ions and negative heparin anions of various sizes. Studies on the chemistry of sodium heparin and related substances have been reviewed by Jaques [5].

This report discusses a method for rendering polysilicone thromboresistant by chemically binding it to heparin. An earlier report described a similar method for chemically binding heparin to polyethylene [10]. In brief, the substrate polymer is radiation grafted with chloromethylstyrene (CMS), quaternized by reaction with pyridine, and then heated with a solution of sodium heparin so that chloride anions in the graft copolymer are replaced with heparin anions.

The coating technique, which is more common than the grafting method, makes use of tridodecylmethylammonium chloride (TDMAC). The sodium heparin and the TEDMAC are first dissolved together in a suitable solvent [3]. The resulting solution is coated on the polymeric substrate. During prolonged exposure to the bloodstream the heparin in the coating undergoes leaching [2, 11-13], which can be slowed down by treating the heparinized surface with glutaraldehyde [14, 15]. It is possible to stop leaching completely by covalently bonding heparin to the polymer substrate, but covalently bound heparin has less anticoagulant effect than the corresponding amount of ionically bound heparin [13]. The method followed in the present research, of radiation grafting followed by heparin reaction, yields ionically bound heparin in a thromboresistant product, which is strongly resistant to heparin extraction by normal saline solution.

EXPERIMENTAL

The technique of radiation grafting used in this work has been discussed in detail [15-19], and will only be summarized briefly. The polymer sample to be grafted is positioned precisely in the cobalt 60 source, thereby allowing reproducible grafting rate measurements to be made. An exposure dose rate at the sample of 310 R/h for the

initial work was measured using ferrous sulfate dosimetry [20]. Later work was done at an exposure dose rate of 270 R/h.

The CMS monomer from Polysciences, Inc., was washed three times with 10% aqueous sodium hydroxide, then three times with distilled water, dried over anhydrous calcium sulfate, distilled at reduced pressure, and stored over anhydrous calcium sulfate at refrigerator temperature. Just before each irradiation run, the portion of monomer containing the immersed polymer sample was deoxygenated by freeze-thaw cycling in a vacuum system. The capsule containing polymer sample and monomer was frozen down in a Dry Ice trap and sealed from the vacuum line with a hand torch, then placed in the ^{60}Co source in the predetermined position.

After each run the grafted sample was extracted in warm benzene for 24 h, a procedure found satisfactory for either polyethylene or polysilicone samples. The purpose of the extraction was to remove CMS homopolymer formed within the sample. All grafting runs were made at the temperature of the irradiation room, 23 ± 1°C. Percent grafting (CMS add-on) was computed from original sample weight (P_0) before grafting, and the weight after drying to constant weight following grafting (P_g) by the use of

$$\% \text{ grafting} = \frac{P_g - P_0}{P_0} \times 100 \quad (1)$$

This grafting technique resulted in the incorporation of chains of CMS units into the grafted polysilicone sample. The chloromethyl groups were quaternized by stirring the grafted sample in pyridine for 24 h at 50°C, followed by drying to constant weight. This procedure is similar to the technique of Stamm [21] for the quaternization of polypropylene grafted with CMS.

The quaternized, grafted samples were next heparinized by placing in a 5.0% solution of sodium heparin (Sigma Chemicals) in a solvent consisting of a 40/60 volume ratio of dioxane/water and holding at 70°C for 24 h, followed by drying to constant weight. The dioxane was included to obtain adequate penetration of the sodium heparin into the (swollen) solid phase. The effect of the dioxane was demonstrated by observing the weight increase (percent swelling) of small polysilicone samples at saturation when immersed in various dioxane/water ratios at room temperature (Table 1). At the same time, the water concentration in the chosen solvent was great enough to cause the sodium heparin to dissolve.

While questions remain about the structure of heparin, it is generally agreed that ionic compounds such as sodium heparin contain heparin anions which are negatively charged due to the presence of sulfate groups [2]. The reaction between the quaternized graft copolymer and sodium heparin probably involves an exchange whereby

TABLE 1. Saturation Weight Increase of Polysilicone in Dioxane/Water Solution at Room Temperature

Dioxane/water, volume ratio	% Weight increase[a]
100/0	25.1
90/10	15.8
80/20	12.8
70/30	10.5
60/40	9.5
50/50	7.5
40/60	5.7
30/70	3.9
20/80	2.1
0/100	0.0

[a]Using polysilicone Code No. 5062 REV2 HH3198.

chloride anions in the copolymer are replaced by heparin anions. The equations hypothesized for the quaternization and heparinization reactions were presented in the earlier report [10]. The heparinization equation can be abbreviated as

$$XSMP^+Cl^- + H^{-X} \longrightarrow [SMP^+]_X H^{-X} + XCl^- \quad (2)$$

where SMP^+Cl^- represents a chain unit of quaternized chloromethylstyrene. If the heparin polyanion bears X negative charges, it can replace X moles of chloride anions. (See discussion below concerning relative amounts of pyridine and heparin add-on.)

RESULTS AND DISCUSSION

If one wishes to obtain meaningful measurements of grafting rates, some account must be taken of the "diffusion effect." This effect involves the limitation of the grafting reaction due to the slowness of monomer diffusion into the solid polymer. Also, in the radiation grafting of a thick sheet of polymer, the monomer reacts and is consumed before it can diffuse to the center of the sheet [22]. The result is that the monomer penetrates into the sample to a limited depth, d,

TABLE 2. CMS Grafting on Polysilicone Rings[a]

Sample no.	Time (h)	CMS add-on (%)	Pyridine add-on (%)	Heparin add-on (%)	Pyridine / CMS wt ratio	H/P wt ratio
32	48.3	9.8	5.1	0.5	0.52	0.10
35	92.8	12.9	6.9	2.9	0.53	0.42
36	115.7	14.5	8.8	4.3	0.62	0.49
40	94.4	12.5	-	-	-	-
41	23.2	7.5	-	-	-	-

[a]Using Dow Corning polysilicone, Code No. 5062 REV2 HH3198.

and all of the grafting takes place within a distance d of the surface [23].

In order to avoid this diffusion effect, the initial runs were carried out on small, thin samples of polysilicone weighing about 0.04 g. Each sample was a thin ring cut from the end of a polysilicone tube of approximately 0.375 in o.d. and 0.250 in i.d. (Dow Corning Medical Products, Code No. 5062 REV2 HH3198). The results of such initial grafting experiments on small samples are summarized in Table 2, which shows percent add-on of CMS, pyridine, and heparin for various irradiation times. A graphical plot of grafting versus irradiation time is presented in Fig. 1, with corresponding percentage add-on of pyridine and heparin shown. (The add-on for both pyridine and heparin was computed on original sample weight.) The heparin add-on curve shows an unpredicted intersection of the time axis at 42 h, instead of passing through the origin. If it is assumed that every CMS grouping in the graft copolymer is quaternized, the ratio of weight gain on quaternization to grafting weight gain should equal the ratio of pyridine molecular weight to CMS molecular weight, namely 79.0/152.5 or 0.52. Table 2 indicates that Samples 32 and 35 fall close to the theoretical value. An unexplained aspect of Fig. 1 is the fast add-on of CMS during the first 20 h of irradiation, followed by a slower, linear add-on between 20 and 120 h. The most common type of monomer/polymer grafting curve exhibits a linear portion passing through the origin.

It is also of interest to consider the heparin/pyridine add-on ratio. If the molecular weight of heparin is about 20,000 [5], it is evident that one can write

$$\frac{20{,}000 \times \text{mol heparin}}{79 \times \text{mol pyridine}} = \frac{\text{wt heparin}}{\text{wt pyridine}} \tag{3}$$

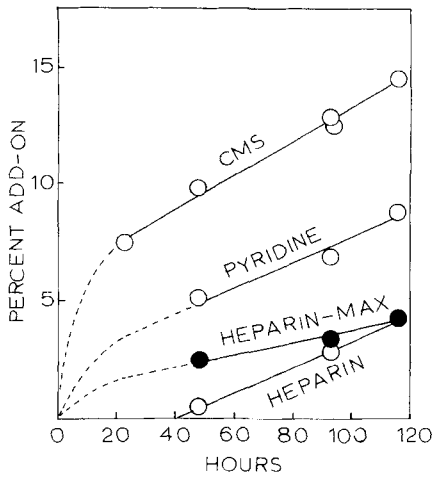

FIG. 1. Room temperature grafting of CMS on small rings of polysilicone (Code No. 5062 REV2 HH3198); subsequent add-on of pyridine and heparin shown; exposure dose rate of 310 R/h. Filled circles are hypothetical values for "heparin-max" computed from pyridine add-on assuming H/P = 0.49.

In Table 2 the heparin/pyridine weight ratio ranges from 0.49 for Sample 36 to 0.10 for Sample 32. This means that the number of molecules of bound pyridine per molecule of bound heparin ranges from 517 to 2500. This agrees qualitatively with the fact that each heparin polyanion bears several hundred negative charges. Any attempt to draw quantitative conclusions is obviated because: (1) sodium heparin penetrates poorly and many of the interior quaternized groupings may never undergo reaction, and (2) even if enough heparin is present to react with all quaternized groupings, there is no evidence to date on whether the reaction is stoichiometric.

Additional grafting runs were carried out on tubular samples of polysilicone, of a size and shape suitable for thromboresistance testing following grafting. Each sample was a tube 4.0 cm long, 0.0655 in. i.d. and 0.1945 in. o.d., cut from a longer tube obtained from Cole-Parmer Instrument Co. (Stock No. 6411-43). Table 3 summarizes percent add-on of CMS, pyridine, and heparin at various irradiation times, employing the techniques discussed above. A plot of percent add-on of CMS, pyridine, and heparin versus irradiation time is shown in Fig. 2. All of the add-on plots are linear up to about 70 h of irradiation, after which the grafting rate declines. The lowest curve, corresponding to heparin add-on, shows an unpredicted intersection of the time axis at about 15 h, instead of passing through the origin.

TABLE 3. CMS Grafting on Polysilicone Tubes[a]

Sample no.	Time (h)	CMS add-on (%)	Pyridine add-on (%)	Heparin add-on (%)	Pyridine / CMS wt ratio	H/P wt ratio
51	21.7	4.6	3.0	0.27	0.65	0.09
58[b]	66.3	14.0	9.1	1.86	0.65	0.20
59	71.0	15.2	9.9	1.93	0.65	0.20
61	45.4	9.9	7.3	0.59	0.73	0.12
62	92.3	15.7	11.1	2.13	0.71	0.19
R1[c]	0.0	0.0	0.0	0.0	-	-

[a]Using Cole-Parmer Instrument Co. tubing, Code No. 6411-43.
[b]Sample 58 was stirred in normal saline solution to determine ease of heparin extraction.
[c]Control sample; untreated polysilicone.

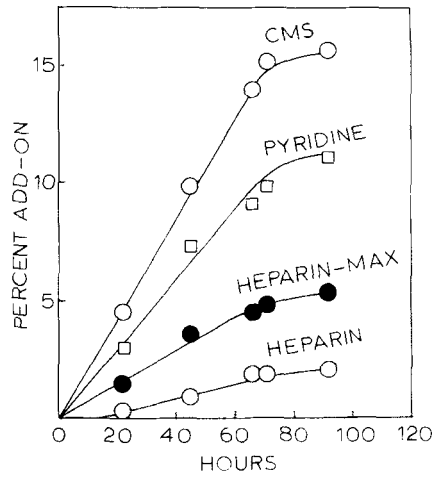

FIG. 2. Room temperature grafting of CMS on 4.0 cm tubes of polysilicone (Stock No. 6411-43); subsequent add-on of pyridine and heparin shown; exposure dose rate of 270 R/h. Filled circles are hypothetical values for "heparin-max" computed from pyridine add-on assuming H/P = 0.49.

Table 3 presents the computed weight ratios for pyridine/CMS, which are considerably larger than the theoretical ratio of 0.52. A possible explanation is that the batch of CMS used in obtaining these data contained a small proportion of monomer molecules bearing more than one chloromethyl group per molecule.

Table 3 also shows that the average heparin/pyridine ratio for tubes is somewhat smaller than the corresponding ratio for small rings (Table 2). This would correlate with a less efficient penetration of sodium heparin into the relatively thick tubes during the heparinization reaction. It is not surprising that a small ring sample, No. 36, shows the highest H/P ratio of any sample studied, corresponding to the most efficient heparin penetration observed in the present work.

Even though the completeness of Reaction (2) is not certainly known for any of the samples listed, it is possible to advance a hypothesis which leads to an interesting interpretation of the data. Assume that the H/P ratio of 0.49 for Sample 36 represents essentially complete penetration of the sample by heparin and corresponds to the maximum possible extent of the heparin reaction under the conditions employed. Hypothetical values for maximum heparin add-on for all of the samples are then computed by multiplying the percent pyridine add-on for each by the constant factor 0.49. Use of the constant factor of 0.49 is equivalent to the assumption that the number of molecules of bound pyridine per molecule of bound heparin equals 517 in all cases. The computed maximum heparin add-on curves are presented in Figs. 1 and 2, with the newly computed points shown as filled circles. The hypothetical maximum heparin curves may approximate the add-on behavior that would have been observed if the heparin had penetrated all samples and reacted completely. The hypothetical curves can be easily extrapolated to pass through the origin in quite reasonable fashion as shown.

In comparable previous work on polyethylene samples, a maximum H/P value of 0.53 was observed [10]. The range of H/P values for all polyethylene samples was from 0.28 to 0.53, indicating efficient penetration by heparin, which is consistent with the fact that the samples weighed only 0.04 g and were only 0.012 in. thick.

An important question which has been discussed but not resolved in the literature relates to whether the heparin must leach gradually from the heparinized polymer in order to produce optimum anticoagulant activity, or even any such activity. For example, Tanzawa [24] stated that the critical value for patency of heparin-coated shunts was a loss rate above 4×10^{-8} g/cm^2/min. Also Schmer [25] concluded that the minimal heparin release rate for the prevention of clotting in the hollow fiber artificial kidney is 1.35×10^{-8} g/cm^2/min. On the other hand, Salzman [26], Merrill [27], and Wong [28] state that the antithrombogenic effect does not depend on the leaching of heparin into the blood, as shown by sensitive clotting tests and studies with radioactive heparin.

The ease of heparin leaching from Sample 58 (1.86% heparin add-on)

was tested by extracting it with normal saline solution (0.9% sodium chloride in water) for 22 h at 50°C. After drying to constant weight, the observed weight loss indicated a maximum possible decrease in heparin content from 1.86 to 1.61%, assuming that all of the observed weight loss was due to heparin removal. The assumption is reasonable, because similar samples grafted with CMS and quaternized with pyridine showed zero weight loss when subjected to the same extraction procedure. Sample 58 was extracted a second time in normal saline solution for 24 h at 50°C. There was a further drop in heparin content to 1.52%, again assuming that all of the observed weight loss was due to heparin removal. The first day's extraction loss of 0.25% and the second day's loss of 0.09% indicate that the rate of heparin loss by normal saline extraction is a decreasing function of extraction time. While these data are not extensive enough to justify firm conclusions, it would be reasonable to hypothesize that continued extraction would cause the sample to lose heparin very gradually over a long period of time.

Since Sample 58 weighed 0.7380 g, the second day's loss of 0.09% corresponded to a loss rate of 6.7×10^{-4} g/d or 4.7×10^{-7} g/min. The sample was 4.0 cm long, 0.494 cm o.d., and 0.1664 cm i.d., indicating a total surface area of about 8.7 cm^2 (outer surface plus inner surface plus two ends). The loss rate from the sample surface during extraction therefore equals $4.7 \times 10^{-7}/8.7$ or 5.4×10^{-8} g/cm^2/min. This rate is of the same order as the preferred leaching rates quoted by Tanzawa [24] and Schmer [25].

The present technique produces ionically bound heparin anions that can move about near and on the surface, but usually cannot escape because they are held by the positively charged chains of quaternized monomer units. The positive cationic chains of grafted monomer are covalently bonded to the polymeric substrate, in which they are literally embedded. Even if the heparin anions should escape from the surface by abrasion or other means, they would be replaced by other heparin anions diffusing from within the interior of the polymeric substrate. Such binding of high molecular weight ions to polymers of opposite charge has been termed "ion-atmosphere binding" by Strauss, and the phenomenon has been discussed extensively by Strauss [29], Reichenberg [30], and Fettes [31]. Covalently bound heparin would have greatly decreased mobility, as noted by Falb [13]. The covalent bonding of the quaternized side chains is a distinct advantage of the present technique, because it prevents their leaching out of the heparinized graft copolymer. One of the disadvantages of the TDMAC type of surface coating has been noted by Eberle [32] and others, who observed toxic side effects in experimental dogs using membrane oxygenator bypass systems coated with leachable quaternary ammonium salts.

THROMBORESISTANCE TESTING

A simple bleeding test was devised in order to determine in preliminary fashion the thrombogenic properties of the heparinized samples. Each of the tubular samples (Table 3) was connected to a modified 18 gauge Angiocath with a sterile Teflon sheath (Deseret) in order to allow an ex vivo bleeding experiment. A 1-cm length of the Angiocath sheath was the only material interposed between the test material and the blood supply. The Angiocath and test material were filled with sterile Ringers lactate, and care was taken to avoid the presence of macrobubbles in the tube preparation prior to insertion. The Angiocath needle was inserted into a rabbit ear vein (New Zealand white, 3.6 kg average weight) with the modified Teflon sheath and attached test sample overlaying the needle. Once the needle was inserted, the sheath was slid into the vein, the needle removed, and the experiment commenced.

Blood was allowed to flow for 2.5 min from the ear vein through the test section of tubing and collected in a clean 50 mL glass beaker. After the blood flow period, the Teflon sheath and that section were removed and rinsed, in a retrograde flow arrangement, with heparinized Ringers lactate at 4 mL/min for 30 min. The time required to form a clot in the effluent blood in the beaker was measured. Samples 58, 59, 61, and 62 were evaluated in this fashion and compared with one additional sample of polysilicone tubing (R1) which had not been treated at all. The reference sample was prepared in the same fashion as the test samples. Following the wash period, all samples were slit longitudinally, inspected visually for adherent thrombus, and superficially examined under the light microscope. Thereafter the samples were prepared for closer examination of microthrombi and adherent platelets. Samples were critical point dried, coated with palladium, and examined in the Jeolco 35M scanning electron microscope (SEM) at 15 kV and 36, 440, and 4400×. Representative photographs of the proximal and distal thirds of the test section were obtained. Photographs were corrected for magnification distorition, and three 1-in.^{2} regions of each photo were analyzed for single and multiple platelet deposits.

Experimental conditions of testing are given in Table 4 for four heparinized samples (Nos. 58-62) and the control sample (R1) of untreated polysilicone tubing. The blood clotted in the Angiocath tip in Sample 62, thus creating a stagnant dilute blood-lactated Ringers suspension for the 2.5-min flow interval. In all other samples blood flow was adequate, and the crude test of beaker clotting time indicated reasonably normal hemostatic function. Visual inspection showed Samples 58, 59, and 61 were free of thrombotic material. Sample 62 had a red thrombus in the midsection of the test material emanating from the Angiocath sheath. The control sample was completely filled with red thrombus.

SEM analysis indicated variable numbers of singly and multiply

TABLE 4. Summary of Thromboresistance Testing Conditions

Sample no.	2.5 min blood flow, (mL)	Tube shear rate (s^{-1})	Post perfusion clotting time (min)	Visual inspection
58	4	11	4	Clear
59	4	11	10	Clear
61	9	24	4	Clear
62	1	1	-	Clot in midsection
R1	2.5	9	Not measured	Red thrombus fills test section

TABLE 5. Density of Wall Platelet Deposits (particles/mm^2)

Sample no.	Single platelets			Aggregates		
	Proximal	Distal	Average	Proximal	Distal	Average
58	566	943	755	188	564	376
59	3523	5599	4561	2013	943	1478
61	4844	5976	5410	755	880	818
62	514	257	386	629	172	401
$\bar{x}$		2777				768
$\sigma\bar{x}$		874				205
σx		2471				579

R1 - Fibrin-red cell matrix completely fills the test section.

adherent platelets on the test surfaces. Multiple platelet deposits, when observed, consisted of small numbers of platelets, usually less than four. The summary of the data (Table 5) indicates that the number of attached platelets was quite variable. There was no correlation with proximal or distal position for either single or multiple platelet deposits. The mean platelet deposit density was 2777/mm^2 ± 874 (standard error of the mean). The mean aggregate deposition rate was 768/mm^2 ± 205. The control surface was completely covered with a fibrin-red cell matrix.

Consideration of these results suggests that the test material is more blood compatible than the untreated polysilicone in the setting

of a crude, short-term ex vivo bleeding test. A careful inspection of all surfaces for fibrin deposits on SEM analysis indicated an absence of fibrin for all samples except No. 61, for which a few very short fibrin strands were visible, overlaying platelet deposits. Heparinization did not prevent adhesion of single or small multiplets of platelets. It did not lead to mass platelet aggregates, although the exposure time may not have been sufficient for development of larger aggregates. Inspection of the single platelets did not indicate the multiple surface attachment sites characteristic of activated platelets. These results are similar to those for platelet deposits on albumin pretreated surfaces [33].

It is interesting that Sample 58 had the lowest platelet deposit density and also the lowest aggregate deposit density, in spite of undergoing two 1-d extractions with normal saline solution. The extraction procedure seemed to have no detrimental effect on the thrombogenic properties of Sample 58, as indicated by the data of Table 5.

Comparison of Tables 3 and 5 shows no obvious correlation between percent heparin content and antithrombogenic effectiveness. This may mean that the heparin concentrations for all samples listed in Table 5 are well above the lower concentration limit for antithrombogenic effectiveness [34].

Grabowski et al. [35] indicate that a species comparison platelet adhesion test conducted on Cuprophan at a shear rate of 986 s^{-1} for 10 min gave the highest platelet adhesion density for the rabbit at 47,200 ± 10,900 platelets/mm^2. In contrast, dog blood produced the next highest adhesion density, 36,400, and human blood produced less than 100/mm^2. The data from the present study cannot be closely compared with those of Grabowski, since time of exposure, shear rate, material, cannulation technique, animal origin and condition, etc. were not matched. However, it can be said, on the basis of Grabowski's findings, that utilization of the rabbit is a rather severe test of the platelet adherent properties of a candidate biomaterial.

It is to be emphasized that these are preliminary tests, conducted after a relatively small amount of protocol development, in which reproducibility of the rabbit bleeding test was uncontrolled. Work remains to be done to establish the ear vein clotting test as a stable and reproducible method for evaluation of biomaterials. Nevertheless, the preliminary studies are deemed sufficient to indicate an improvement in thrombogenic performance of the radiation-grafted heparinized polysilicone in comparison with the untreated control.

A noteworthy event relevant to this research is the isolation by Rosenberg and Lam [36] of an active heparin fraction making up one-third of the mass of the starting material but responsible for 85% of its anticoagulant activity. Plans are being made to repeat the present work on heparinization of polysilicone using the "active" fraction, and test the resulting products for thromboresistance. This approach is important in terms of the need to minimize the amount of heparin, pyridine, and chloromethylstyrene added to the original polysilicone

substrate. Minimizing the add-on of these components should minimize any possible toxic effects, and also minimize the gradually increasing stiffness which often parallels increasing amounts of grafting. It is also desirable to eliminate the inactive two-thirds of the original heparin because such material, having little anticoagulant activity, could possibly interact with other blood components such as platelets and might endow heparinized polysilicone with undesirable thrombogenic properties.

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