J. Sci. I. R. Iran

Vol. 1, No. 2
Winter, 1990

PREPARATION AND APPLICATIONS OF FLUORESCENT POLYGLUTARALDEHYDE IMMUNOMICROSPHERES

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Keywords: Immunomicrospheres, Polyglutaraldehyde, Brucella, Cell targeting

Abstract

Aqueous glutaraldehyde has been polymerized under alkaline conditions in the presence of a surfactant to yield microspheres of varied diameters. Microbeads of a nominal 300 nm diameter, made fluorescent via fluoresceinisothiocyanate, were used as a carrier of rabbit anti-human red blood cell antibodies. Specific labeling of the human red blood cells as well as diagnostic use of these fluorescent immunomicrospheres in detection of brucella antibody or antigen has been clearly demonstrated.

Introduction

An improvement in the therapeutic efficacy of drugs by selective targeting to a specific site is of clinical importance. Towards this end a recent development has been the use of targeted drug delivery via immunotoxins (i.e. drug-antibody molecular conjugates) [1]. Despite the success achieved in direct coupling of drugs to antibodies this approach suffers from the fact that only a small number of functional groups are available per antibody molecule which can be successfully used without significant loss of antibody activity. This effectively limits the molar drug-to-antibody ratio to 10:1. To increase this ratio, drugs have been conjugated to carrier molecules such as dextran and human serum albumin [2,3]. The use of drug-loaded polymeric microspheres coated with antibody (immunomicrospheres) may circumvent these problems, and in fact microbeads carrying drugs up to 50% of their weight have been reported [4].

Antibodies (immunoglobulins) are bifunctional molecules which not only bind to antigens, but also initiate a number of other biological phenomena such as complement activation and histamine release by mast cells, activities in which the antibody acts as a directing agent.

These two kinds of functional activities are localized to different portions of the molecule: the antigen-binding activity to Fab and the biological activities to the Fc portion of the molecule. Structurally they have a tetrameric arrangement of pairs of identical light and heavy polypeptide chains held together by noncovalent forces and usually by interchain disulfide bridges. Each chain is made up of a number of loops or domains of more or less constant size. The N-terminal domain of each chain has greater variation in the amino acid sequence than the other regions, and it is this which imparts the specificity to the molecule. There are five types of heavy chains, which distinguish the class of immunoglobulins IgM, IgG, IgD, IgA, and IgE, and two types of light chains. In many species there is often more than one version of some of the heavy chain classes, which impart distinct physicochemical and biological characteristics to the molecule [5].

The introduction by Kohler and Milstein [6] of methods for producing hybrid myelomas (hybridomas) that synthesize monoclonal antibodies against single antigenic determinants has allowed the production of

apparently inexhaustible suplies of pure, specific, and standardized antibodies.

Immunomicrosperes

Monoclonal antibodies can be attached to polymeric microspheres by means of direct coupling if functional groups capable of covalently bonding with proteins, e.g. aldehyde groups, are available on their surface. Polymeric microspheres can be made from many different materials in many different ways in order to obtain functional groups (carboxyl, ester, hydroxyl, aldehyde, sulfonate, amino, and amide).

The so-called latex polymer systems are made by an aqueous polymerization process. For example, polystyrene latex particles possessing a variety of reactive surface groups can be prepared without emulsifier using persulfate initiator and bicarbonate buffer and employing proper after-treatments.

Reactive groups can also be incorporated into the microspheres by an emulsion copolymerization process. Such reactive groups can be used for the binding of antibodies either through a coupling reagent or modification to reactive aldehyde groups.

It is therefore, clear that microspheres with native aldehyde groups are particularly desirable, since the attachment of antibody molecules to them takes place automatically in one simple step (reaction between the aldehyde group and a primary amine group on the protein molecule). Thus, in this work we have investigated the use of polyglutaraldehyde microspheres in cell labeling via attaching corresponding antibodies to their surfaces.

Experimental Section

All chemicals used were analytical grade obtained from commercial suppliers.

1.Polyglutaraldehyde (PGL) Microspheres

PGL Microspheres were prepared following a modification of the method described by others [7]. Briefly stated, the method is as follows: a 5% aqueous solution of glutaraldehyde was polymerized in a small vial at pH=11 while being stirred in the presence of a proper surfactant like Tween 80*. The average size of PGL microspheres can be varied by changing the surfactant concentration (proper and constant stirring condition). Fluorescent PGL microphores were prepared by adding 11% (V/V) fluoresceinisothiocyanate in ethylene diamine (1/20 W/V) to the polymerizing batch.After12-18 hours polymerization was essentially complete and the size of the particles were determined by examining them under an optical microscope. In the work described here, only microspheres of 300nm nominal diameter were used. Water content of the microspheres was determined by weighing the blotted pellets obtained after their centrifugation and their corresponding dried samples obtained in a vacuum oven.

2. Antibody Preparation

Rabbit immunoglobulin against the human red blood cell (RBC) was obtained following standard procedures [8]: Human RBC's were collected via centrifugation of human blood at 1000g, followed by 10X dilution with phosphate buffer saline (PBS) of pH=>7.2. RBC samples were then injected into the rabbit at intervals and after a period of 5-6 weeks (required for the appearance of the antibody) blood

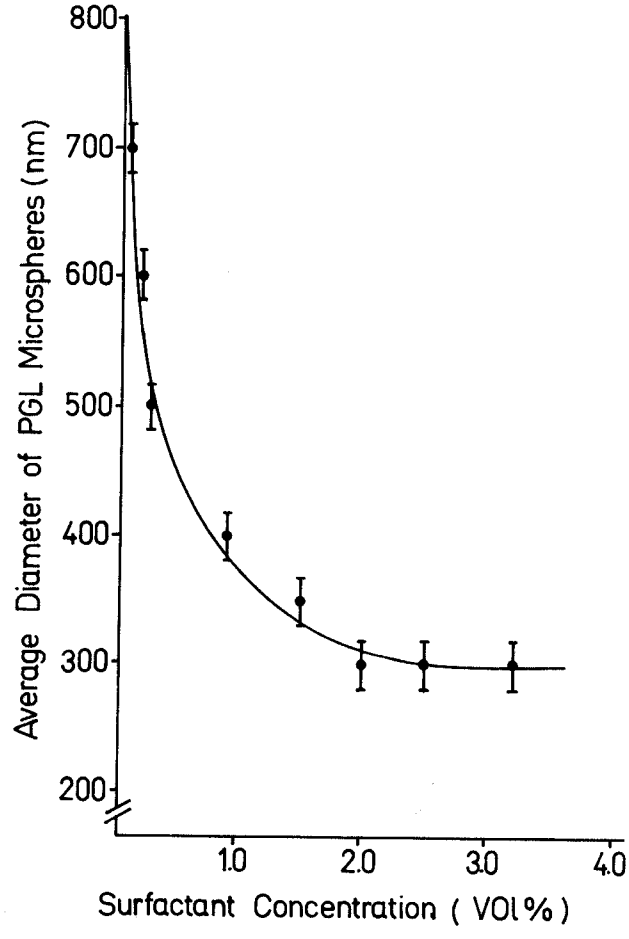

Fig.1. Microsphere diameter as a function of surfactant concentation.

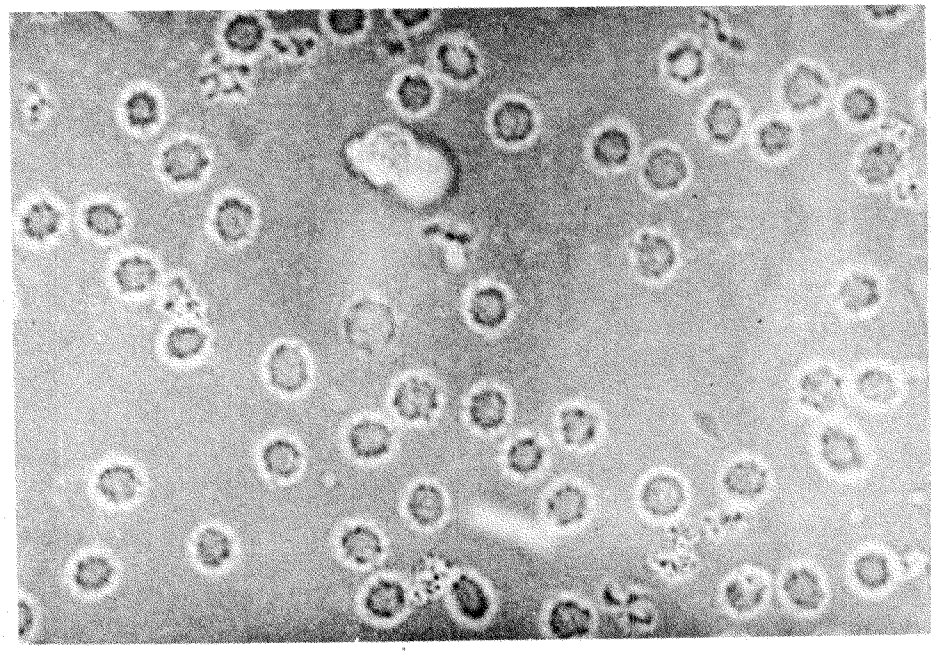

Fig.2. A view of fluorescent immunomicrospheres surrounding human red blood cells.

samples were withdrawn from the animal. Anti-human RBC antibody was precipitated from the rabbit blood serum (the latter obtained by centrifuging the animal blood at 1000g) by adding 40% and 45% ammonium sulfate to the serum. The protein concentration in the samples were determined via absorbance measurement at 280nm.

3. Immunomicrospheres

To a 2 mg sample of PGL microspheres were added 0.2 mg of the antibody and 0.5ml of PBS and the mixture was incubated at 4°C on a shaker for 2hrs. Subsequently 10 mg of urea was added and incubation was continued for another hr. while shaking at room temperature. The mixture was then centrifuged at 750g for 15 min. and the pellets obtained were washed twice with PBS and finally suspended in 0.5 ml of PBS.

Results

Water content of the PGL microspheres was found to be about 60% (Wt). Figure (1) depicts the effect of surfactant concentration on the average size of PGL microspheres. Figure (2) shows the human RBC's surrounded and labeled by the fluorescent PGL immunomicrospheres, as seen through a fluorescent microscope. Figure (3) represents a possible application of the PGL microspheres in clinical diagnostics. Here the microbeads carrying brucella antibodies demonstrate a visible agglutination upon encounter with their corresponding antigen.

Discussion

Figure (1) shows the expected reduction in particle size with an increase in surfactant concentration. As polymerization proceeds and heavier molecular species are generated, their solubilities in water decrease, hence they are kept in suspension in the form of small spheres by the surfactant molecules present.

Although no one yet seems to have specifically investigated the polymerization mechanism involved, it is expected and quite likely that it proceeds essentially through an aldol polycondensation reaction (see Fig. 4). If that is indeed the case, a complex crosslined polymeric network carrying conjugated and nonconjugated aldehyde groups, will result (this has been confirmed by IR spectroscopy).

Based on these arguments the following mechanism seems plausible: The abundance of reactive aldehyde groups within the PGL network makes it highly suitable for direct attachment of antibodies as well as other proteins. Our experience shows that the PGL microspheres are very reactive towards various

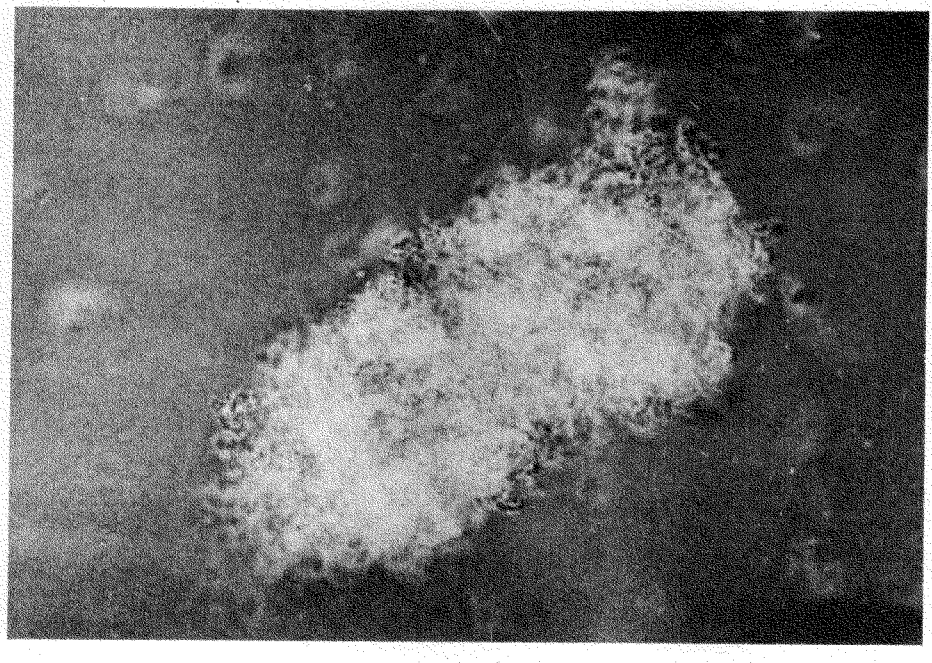

Fig.3. Agglutination of immunomicrospheres in the presence of brucella antigen.

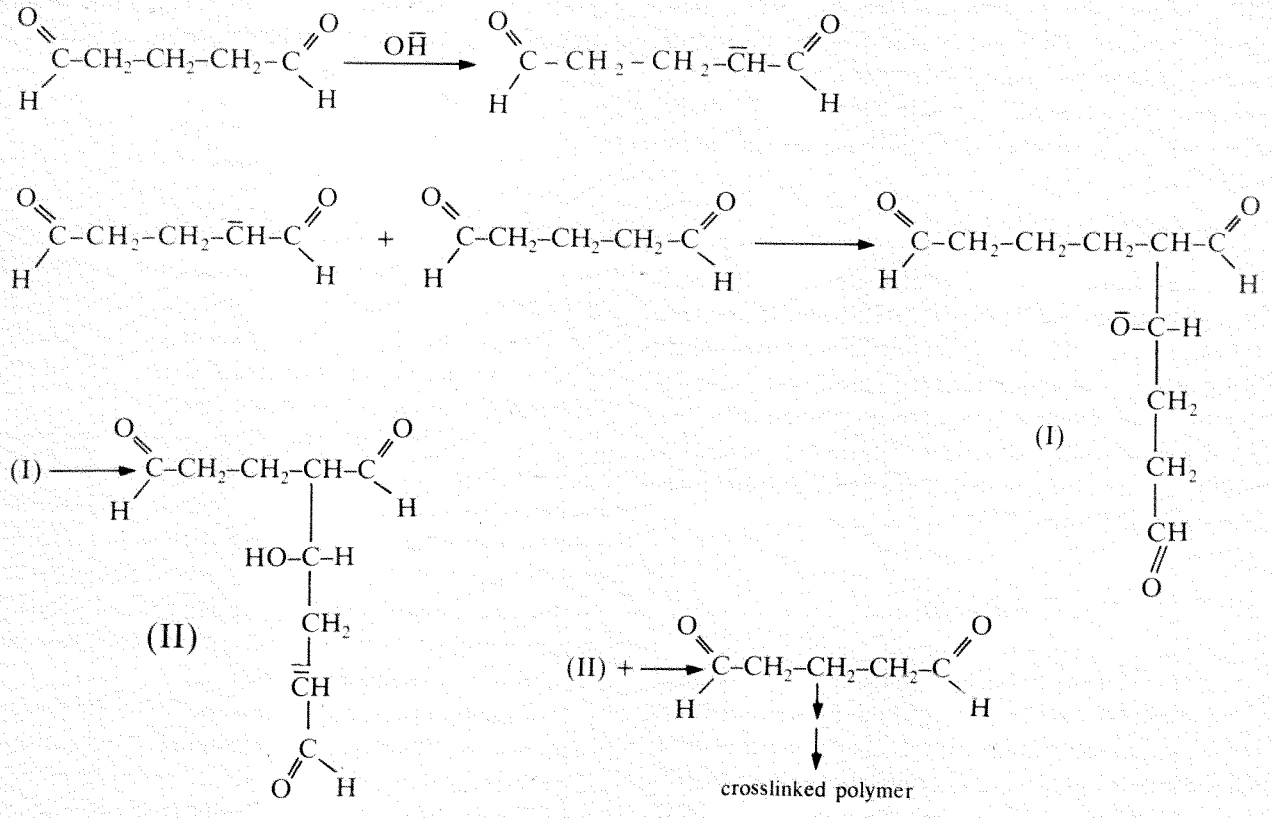

Fig.4. Proposed polycondensation mechamism

enzymes, being capable of immmobilizing them without significant alteration of their activities.

A unique feature of the PGL microspheres is the fact that attachment of the enzymes or antibodies can be accomplished in one simple step, without any prerequisite treatments. There are however, some drawbacks about the PGL microspheres that are worth mentioning. First and foremost is their strong tendency to self agglomerate upon long time storage. Such event is not surprising considering the gel like structure (60% water) of these microspheres. Second, is the toxicity associated with the unreacted glutaraldehyde, which if properly washed might be minimized.

We therefore conclude that a superior approach would be to have a composite microsphere made of a rigid core of an inert hydrophobic polymer (e.g. polystyrene), coated with a thin layer of PGL. It is our hope to develop such microspheres in our future acitvities.

Acknowlegement

The funding for this research was provided by a grant from Tehran University's Council of Research (Grant No. 558).

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