SYNTHESIS, PROPERTIES AND BIOMEDICAL APPLICATIONS OF HYDROPHILIC, FUNCTIONAL, POLYMERIC IMMUNOMICROSPHERES

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Abstract - Immunomicrospheres consist of hydrophilic cross-linked particles with antibodies bound to their surface. These particles find specific receptors on living or fixed cells and therefore can label cell sub-populations and the labelled cells can be observed by means of a scanning electron or light-microscope. To label cells with polymeric particles, biocompatible microspheres are needed preferably with a narrow distribution of sizes in the range of 10 to 10,000 Å. Several classes of hydrophilic cross-linked microspheres with functional groups e.g. carboxyl, hydroxyl, amide and/or pyridine groups were synthesized. These functional groups were used to bind covalently antibodies and other proteins to the surface of the microspheres. To optimize the derivatization technique, polyglutaraldehyde immunomicrospheres have been prepared and utilized. Specific populations of human and murine lymphocytes were labelled with microspheres synthesized either by the emulsion or the ionizing radiation technique. The labelling of the cells be means of microspheres containing an iron core led to a successful separation of B from T lymphocytes by means of a magnetic field.

INTRODUCTION

The progress of medicine and biology depends to a large extent on our knowledge of the structure and properties of antigens, or cell receptors, which are exposed on the surface of the cell outer membrane (1). Differences in cell surface antigens are also widely used for cell classification and separation. For these reasons, intensive efforts were made in the past to detect, characterize and quantitate these antigens. In recent years, attention has been focused on the study of antigens by immunological methods, i.e., by coupling antibodies to either radioactive isotopes, fluorescent dyes, biological macromolecules or synthetic particles (2-6). These methods have been applied successfully to the identification of various cells, for example, to subpopulations of B and T lymphocytes (7-9) and neural and Schwann cells (10).

The practical applications of these investigations are diagnostic tests of a large number of diseases and their therapy. Unfortunately, the previously used reagents suffer from either lack of stability, nonspecific interactions or restricted dimensions. For example, ferritin, a well known cell surface marker, useful for electron microscopic studies (11), cannot be employed to distinguish cell subpopulations under the light microscope.

Recently, we synthesized polymeric microspheres chemically coupled to antibodies, i.e, immunomicrospheres as new reagents for cell mapping and separation. The immunomicrospheres are relatively stable, easily obtainable in a wide variety of sizes and useful for flow cytometry since they can bind specifically to selected cells and are detected by light scattering or fluorescence (12).

They may be used in conjunction with other biological stains if desired (13, 14) and can be visualized by fluorescence or light microscopy as well as with the scanning electron microscope (SEM) (15, 16). Apart from cell receptor mapping, they may find important applications in cell separation. There is little doubt that physical sorting of cell subpopulations has become a necessity. New flow cytometer and sorters permit quantitative measurements but are limited with regard to the number of cells that can be separated in a given gime (17).

Immunomicrospheres of suitable composition change the electrokinetic behavior of living or aldehyde fixed cells, thus allowing their separation by means of recently developed continuous flow electrophoretic instruments (18). B lymphocytes labelled with iron or magnetite containing immunomicrospheres were separated by means of a magnetic field (19). The magnetic separation was also successfully applied to neuroblastoma cells (20). With the advent of highly specific monoclonal antibodies, these new reagents therefore may yield improved labelling procedures and exciting new applications in biology and medicine, including currently very difficult separation of specific cells occurring only in small numbers (e.g., fetal cells in peripheral blood). Similar techniques could also be used to isolate specific

antigens for biochemical and immunochemical studies.

APPROACH

It is possible to distinguish cell subpopulations which have different antigens on their surface by labelling the cells with immunomicrospheres to which specific antibody is covalently bound. A direct or indirect technique may be used. In the direct technique a microsphere to which an antibody is covalently bound seeks out the cell antigen and binds to it and in the indirect labelling procedure an intermediate antibody is employed. The first step of both procedures requires the covalent binding of a purified antibody to a microsphere through functional groups on its surface.
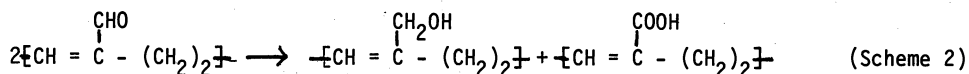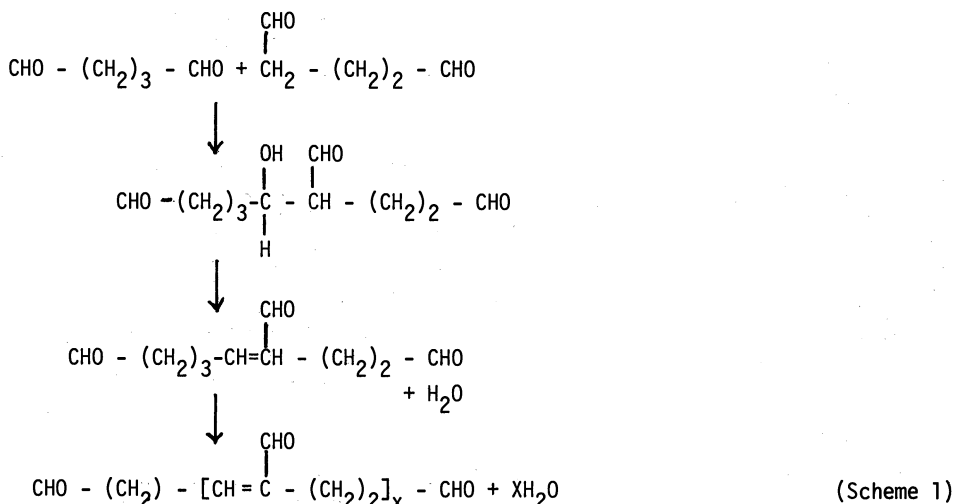
SYNTHESIS AND DERIVATIZATION OF FUNCTIONAL MICROSPHERES

Several classes of hydrophilic functional microspheres have recently been synthesized using either the emulsion or ionizing radiation technique. (For experimental details, characterization and applications see references 21, 22)
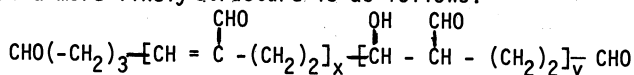
The main advantage of the ionizing radiation technique is that microspheres practically free of impurities are obtained, since the synthesis is carried out in the absence of free radical initiator or emulsifier. Both techniques, however yield particles which, depending on the initial monomer composition, have either carboxyl, hydroxyl, amide or all three functional groups on their surface. These can therefore be utilized to covalently bind proteins according to well established methods (23). A kinetic study of covalent binding of antibodies to solid particles showed (22) that the glutaraldehyde reaction was faster than either the cyanogen bromide or carbodiimide reaction. Since glutaraldehyde undergoes aldol condensation during the process of antibody binding and also in order to simplify the derivatization procedure the possibility of synthesis of polyglutaraldehyde (PGL) microspheres was investigated.

POLYMERIZATION OF GLUTARALDEHYDE AND SYNTHESIS OF PGL MICROSPHERES

Glutaraldehyde polymerizes in aqueous solution, and its polymerization rate depends on the pH, concentration and temperature. The polymerization of glutaraldehyde in the presence of surfactants yielded microspheres of various sizes, depending on the reaction conditions (24). As anticipated, a one step room temperature reaction with antibodies yielded immunomicrospheres which were utilized to label human rbc. The mechanism of glutaraldehyde polymerization is represented by Scheme 1 and 2.

$$CHO - (CH_2)_3 - CHO + \underset{\underset{CH_2}{|}}{\overset{\overset{CHO}{|}}{}} - (CH_2)_2 - CHO$$

$$\downarrow$$

$$CHO - (CH_2)_3 - \underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}} - \overset{\overset{CHO}{|}}{CH} - (CH_2)_2 - CHO$$

$$\downarrow$$

$$CHO - (CH_2)_3 - CH = \overset{\overset{CHO}{|}}{CH} - (CH_2)_2 - CHO$$
$$+ H_2O$$

$$\downarrow$$

$$CHO - (CH_2) - [CH = \overset{\overset{CHO}{|}}{C} - (CH_2)_2]_x - CHO + XH_2O \qquad \text{(Scheme 1)}$$

$$2[CH = \overset{\overset{CHO}{|}}{C} - (CH_2)_2] \longrightarrow [CH = \overset{\overset{CH_2OH}{|}}{C} - (CH_2)_2] + [CH = \overset{\overset{COOH}{|}}{C} - (CH_2)_2] \qquad \text{(Scheme 2)}$$

The aldol condensation involves a dehydration step yielding ethylene linkages conjugated with aldehyde functions. According to chemical, spectroscopic and electrochemical analysis the number of aldehyde and double bonds conjugated with the aldehyde groups varied as a function of pH and their concentration was considerably less than theoretically calculated. Therefore a more likely structure is as follows:

$$CHO(-CH_2)_3[CH = \overset{\overset{CHO}{|}}{C} - (CH_2)_2]_x [\overset{\overset{OH}{|}}{CH} - \overset{\overset{CHO}{|}}{CH} - (CH_2)_2]_y CHO$$

This structure implies that dehydration does not always occur at every consecutive step of the aldol condensation reaction at room temperature and would account for the reduced concentration of double bonds conjugated with aldehyde functions. The decrease of the number of aldehyde functions was proven to result from a Cannizzaro reaction (Scheme 2) most probably occurring intermolecularly since the intramolecular mechanism would require an eight membered intermediate (25).

The high reactivity of PGL, whether in solution, in solid state or in the form of water insoluble microspheres offer possibilities for important applications of PGL as a new immunoreagent in the biological and biomedical fields (26).

APPLICATIONS OF POLYMERIC MICROSPHERES AS VISUAL MARKERS FOR LIGHT AND SCANNING ELECTRON MICROSCOPY

Immunomicrospheres have been used to identify specific populations of cells by fluorescent light and SEM and to study the distribution of antigens on cell surfaces. In general, the indirect immunological labelling technique has been used most frequently. The method involves labelling the cell with antibodies (immunoglobulins) directed against specific cell surface antigens. These cells are then relabelled with immunomicrospheres consisting of microspheres coupled to anti-immunoglobulin antibodies (13-17).

ELECTROPHORETIC CELL SEPARATION

The electrophoretic mobility of fixed human red blood cells immunologically labelled with polymeric 4-vinyl pyridine (PVP) or polyglutaraldehyde (PGL) microspheres was altered to a considerable extent i.e., from -1.07 μm $S^{-1}v^{-1}cm^{-1}$ to -0.65 μm $s^{-1}v^{-1}cm^{-1}$ for human rbc labelled with PVP microspheres and -0.62 μm $s^{-1}v^{-1}cm^{-1}$ for human rbc labelled with PGL microspheres. This observation was utilized in the preparative scale electrophoretic separation by continuous flow electrophoresis (CFE).

Two model systems were used: a) a mixture of labelled and unlabelled human rbc and b) a mixture of labelled human rbc and unlabelled turkey rbc. The labelled cells of both systems (ratio 1:1) were efficiently separated (18) by means of a CFE instrument. It is therefore suggested that resolution in electrophoretic separation of cell subpopulations currently limited by finite and often overlapping mobility distributions may be significantly enhanced by immuno specific microsphere labelling of target populations.

MAGNETIC CELL SEPARATION

A recent new development of importance for cell sorting is the synthesis of functional microspheres containing Fe_3O_4. Preliminary investigations (19) show that T and B cells labelled with magnetic immunomicrospheres can be efficiently separated by means of a permanent magnet.

The separation of human rbc labelled with magnetic PGL microspheres was achieved in the following way: Mixtures of human rbc with the following ratios of unlabelled to labelled cells was prepared; 1:1, 7:1, and 9:1. The mixtures (10 ml) were gently stirred in a glass vial fitted with a horseshoe magnet (300 gauss). At the end of 2 h, cells which were not attracted to the vessel walls were isolated. Cells attracted by the magnet were diluted with 10 ml of PBS and the magnetic separation was repeated. SEM examination showed that 96% of unlabelled cells could be thus separated from all 3 synthetic mixtures (24).

The evidence obtained so far using model cell systems indicates that magnetic PGL immunomicrospheres of desirable sizes can be conjugated with proteins in a single and convenient manner, therefore offering a potential for large scale immunological cell sorting.

OTHER APPLICATIONS

Reagents designed to facilitate the examination of cell surfaces and the separation of cell subpopulations should lead to new areas of investigation in biological research. Sufficiently small electron-dense metal containing microspheres should provide higher spatial resolution of cell surface features than is now possible. It is anticipated that gold or platinum particles incorporated in a polymeric matrix with functional groups on the surface capable of forming a covalent bond with proteins would offer more stable labels than gold particles with physically adsorbed antibodies that are presently used for cell labelling. Gold and platinum microspheres can be very simply obtained by reaction of chlorauric or chloroplatinic acid respectively with PVP microspheres (15) or by polymerization of suitable monomers in presence of freshly formed small colloidal gold particles.

PVP microspheres reacted with chloroplatinic acid, after intravenous injection into mice may be easily detected in electron microscope photomicrographs of spleen, liver and kidney tissue sections.

Therefore, these reagents offer the possibility of analysis of localized pathologic and pharmocologic processes in experimental systems, using ultrastructural techniques. They may also yield information in the studies of the pathology of injurious agents which are introduced in vivo, e.g., particulate lung irritants.

PVP microspheres also form strong charge transfer complexes with negatively charged acceptors, e.g., with dinitrofluorobenzene in which case black PVP complexes are formed. These have been already used to detect inflammatory responses in mouse lungs after the latter was immunized by skin painting with dinitrofluorobenzene (27).

Microspheres are also suitable for studies of phagocytosis, which can be quantitated by flow cytometry. Preliminary experiments have shown, for example, that phagocytosis is retarded by beads (0.8 μ in diameter) carrying pyridine or amide groups (28).

Particularly exciting applications can be anticipated in the area of tumor biology: as far as it can be determined, virtually all cancer cells express an altered cell surface and this phenomenon is a common denominator for a diverse array of tumor types. Since the immuno-microspheres are designed for use in studying cell surfaces, they may find applications in early cancer detection, diagnosis and perhaps new types of therapies.

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