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Gold nanoparticles: preparation, functionalisation and applications in biochemistry and immunochemistry

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Abstract. The review summarises data on the synthesis and functionalisation of gold nanoparticles and their applications in biological investigations. Particular attention is given to applications of colloidal gold in solid-phase assays, immunoassay and studies of biologically active compounds by vibrational spectroscopy. A special section deals with the use of gold nanoparticles as antigen carriers in immunisation. The bibliography includes 406 references.

I. Introduction

Gold was one of the first metals discovered by humans, and the history of its study counts several thousands of years. First data on colloidal gold (CG) can be found in treatises by Chinese, Arabic and Indian scientists, who prepared CG and used it, in particular, for medical purposes as early as 5 – 4th centuries B.C. In the Middle Ages, alchemists in Europe actively studied and used CG. Probably, wonderful colour changes that accompany condensation of gold atoms prepared by reduction of salt solutions led alchemists to believe in transformations of elements, CG being considered as a panacea.[1] For example, Paracelsus wrote about the therapeutic properties of '*quinta essentia auri*,' which was prepared by reduction of gold chloride by ethanolic extracts of plants. In 1583, the doctor of the French king Louis XIII, the alchemist David de Planis-Campy, recommended the use of a colloidal solution of gold in water as an elixir of longevity. The first book on colloidal gold preserved to our days was published by the philosopher and doctor of medicine Francisco Antonii in 1618.[2] This book contains information on the formation of CG and its medical uses, including practical advices. From the mid-17th century, CG was used for the production of red (ruby) glasses, decoration on porcelain (purple of Cassius) and silk colouration.

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The beginning of scientific research on CG dates back to the mid-19th century, when Michael Faraday published an article[3] devoted to methods of synthesis and properties of CG. In this article, Faraday described, for the first time, aggregation of CG in the presence of electrolytes, the protective effect of gelatin and other high-molecular-mass compounds and the properties of thin films of CG. Colloidal gold solutions prepared by Faraday are still stored in the Royal Institution of Great Britain in London.

In the late 19th century – early 20th century, Richard Zsigmondy[4,5] published a series of fundamental papers on the properties of CG. He was the first to describe methods of synthesis of CG with different particle sizes with the use of hydrogen peroxide, formaldehyde and white phosphorus as reducing agents and report on important physicochemical (including optical) properties of gold sols. Zsigmondy used colloidal gold as the main experimental object when inventing (in collaboration with Siedentopf) an ultramicroscope. In 1925, Zsigmondy was awarded the Nobel Prize in Chemsitry 'for his demonstration of the heterogeneous nature of colloid solutions and for the methods he used, which have since become fundamental in modern colloid chemistry'.

Studies by the Nobel Prize laureate Theodor Svedberg on the preparation, analysis of mechanisms of colloidal gold formation and sedimentation properties of CG (with the use of the ultracentrifuge he had invented)[6] are also among classical studies. Svedberg investigated the kinetics of reduction of gold halides and formulated the main concepts about the mechanism of formation (chemical condensation) of colloidal gold particles.

Later studies were concerned with various procedures for the synthesis of gold sols based on both disintegration of metallic gold by electric arc and (primarily) synthesis of colloidal particles from gold halides with the use of chemical reducing agents or irradiation.[7–11]

Nowadays, colloidal gold is used by scientists as a perfect subject for studies of the optical properties of metal particles[12–15] and fractal clusters[16] and the mechanisms of aggregation and stabilisation of colloids.[17–21] Colloidal gold is also applied in analytical chemistry,[22,23] geomicrobiology, geobiochemistry[24] and photography.[25] Colloidal gold, unlike crystalline gold, shows a pronounced catalytic activity.[26] Colloidal gold immobilised on an electrode surface is used in investigations of catalytic processes and electron transport in biomacromolecules.[27]

Examples of the application of CG in medicine, in particular, in colour reactions for proteins which are present in the cerebrospinal fluid and blood serum are documented.[28,29] Methods

developed for these purposes are based on the properties of proteins to serve (depending on their physicochemical properties) as either flocculants or stabilisers of colloidal gold particles. Methods for the treatment of rheumatoid arthritis with CG solutions were developed.[30] Colloidal solutions of the ^{198}Au isotope (the half-life is 65 h) are successfully used for therapeutic purposes in oncology.[31] Non-radioactive CG has found wide application in the diagnosis and treatment of cancer diseases.[32–35]

Gold particles are used for studying the transport of substances into cells by endocytosis,[36] the delivery of genetic material to cell nucleus by biolistic transfection[37] and targeted drug delivery.[38]

However, colloidal gold is most widely applied in medicine as an immunochemical marker. An ideal marker should be readily distinguishable with an electron microscope, have a specified size, shape and structure, be stable against aggregation, be strongly bound to biomolecules and not decrease their activity. Colloidal gold meets all these requirements. For the first time, colloidal gold conjugates with immunoglobulins were used as immunochemical markers in 1971.[39] After the publication in 1973 of the study by Frens[40] concerned with methods of synthesis of CG with a desired particle size, CG-based biospecific markers have found wide application in different fields of biology. Abundant information on the most important aspects of the preparation and application of CG-based immunochemical markers in biology and medicine was summarised in monographs[41–43] and reviews.[44–47]

A colloidal gold particle consists of the crystalline nucleus $[Au]_m$ and $AuCl_4^-$ ions adsorbed on the surface of the nucleus. These ions determine the negative charge of colloidal gold particles, compose the inner layer of the ionic (electric) double layer and determine the adsorption potential. The H^+ ions are present in an intermicellar solution (some of them are located in the adsorption region; other, in the diffuse region of the electric double layer). The gold micelle can be represented as follows:

$$\{[Au]_m|\, n(AuCl_4^-)(n-x)H^+|\, xH^+|\}\,,$$
$$\delta_0 \qquad d$$

where $[Au]_m$ is the micelle nucleus (m is the number of gold atoms, which can vary from several hundreds to millions), n is the number of adsorbed $AuCl_4^-$ ions ($n \ll m$), δ_0 is the adsorption layer thickness and d is the thickness of the diffuse region of the electric double layer.[48]

A high electron density, the ability to scatter and emit secondary electrons, the characteristic absorption and scattering in the visible region of the electromagnetic spectrum and the intense red colour of gold-containing markers enable easy detection of gold particles by different physicochemical methods (microscopy,† photometry, flow cytometry, *etc*.). The possibility of preparing gold sols with different particle sizes and a narrow particle size distribution provides a high resolution of these methods and, in addition, enables simultaneous labelling of two or more antigens (or other ligands). After the corresponding immunochemical reaction, the size of CG particles can be increased by silver[49] or gold[50] enhancement (autometallography), which substantially extends the scope of these methods.

In the last 10–15 years, metal nanoparticles (particularly, gold nanoparticles) have found increasing application as efficient optical transducers of the results of biospecific interactions (for example, of an antibody with an antigen adsorbed on CG particles) into the detected optical signal in devices called biochips and biosensors.[51] These devices are based on the unique optical properties of CG, in particular, the surface plasmon resonance (SPR, see below).[52] The changes in physicochemical parameters of biochips and biosensors that occur in the course of biospecific reactions are detected with the use of visual observations, light-scattering methods, vibrational spectroscopy, *etc*.

Biospecific interactions of biomolecules adsorbed on gold nanoparticles in systems, where nanoparticles exist as ordered self-assembled structures (thin films)[53] or are encapsulated in polymeric matrices,[54] have been extensively studied. Such structures are popular for the detection of biomolecules and microorganisms, for the design of DNA chips, *etc*. In these systems, the optical signal from the marker is sharply enhanced due to a strengthening of the local excitation field in an aggregate formed from gold nanoparticles.

Of nanosize structures, which are the subject of numerous fundamental studies, clusters are of particular interest. They occupy an intermediate position between individual atoms and solids and exhibit properties different from those of both atoms and solids. In particular, their physical characteristics substantially depend on the type and number of atoms involved in these structures, and this dependence becomes weaker as the number of atoms increases, which is indicative of the transformation of the material from the cluster to bulk state (quantum-size effects). Among clusters of simple substances, metal clusters (gold nanoparticles is a typical example) are of particular interest. These compounds attract considerable attention due to the characteristic features of their electronic structures and the relative simplicity of their preparation.

The present stage of development of physics of metal clusters dates back to 1993, when the electron shell structure of these clusters was discovered.[55,56] This structure resembles in many aspects the shell structure of atoms. The experimental discovery of electron shells in polyatomic clusters was almost simultaneously confirmed by theoretical methods.

The theoretical approach was based on the property of metal valence electrons to leave the atoms (to be delocalised) and form a conduction band. It appeared that it is these shared electrons that determine the energy structure of the cluster and its unusual collective properties.[57] In particular, delocalised electrons in metal clusters have an effect on the behaviour of clusters in interactions with external fields. Recent extensive studies of interactions of metal clusters with electromagnetic fields revealed giant maxima (resonances) in electromagnetic absorption spectra. These resonances are associated with excitation of collective vibrations of the electronic system analogous to plasma vibrations of the electronic gas in the plasma and macroscopic metal bodies. These vibrations are called plasmon vibrations; the resonance, the surface plasmon resonance. The amplitude and the frequency range of plasmon resonance in clusters differ from those in macroscopic crystals.

If particles form aggregates, the corresponding plasmon peak is shifted to longer wavelengths or is broadened due to dipole–dipole interactions of light-induced dipole moments of particles in aggregates.[58] Therefore, optical excitations in aggregates composed of metal nanoparticles have a collective nature. As a result, local optical fields acting on metal particles in a fractal aggregate can be substantially higher than the mean field, which leads to giant non-linear effects.[59]

It is of principal importance that the optical response of nanoparticles or their aggregates (particularly, ordered) substantially depends on the particle size and shape, the interparticle distance and the local dielectric environment of the particles.[60] This enables the tuning of the optical parameters of sensors.

The design and practical applications of biosensors based on metal nanoparticles were considered in reviews.[51,61–68] New unique technologies, in particular, the monolayer self-assembly of metal particles,[69] nanolithography,[70] vacuum evaporation,[71] *etc*., are employed in the design of biosensor devices. Biosensors based on gold nanoparticles are used in immunoassay[72] and for the nucleotide sequence determination.[73,74] Recently, record sensitivities (in a zeptomole range) of such sensors have been achieved,[75,76] and it has been demonstrated that resonance scattering spectra of individual particles can be recorded. This

† In addition to conventional transmission electron microscopy, scanning electron microscopy, light microscopy and different modifications of atomic force microscopy are also widely used.

opens up the possibility of observing intermolecular interactions at the single-molecule level.[77]

The unique optical properties of silver and gold nanoparticles, which are determined by the localised surface plasmon resonance, are nowadays widely applied in single-electron transistors,[78] near-field optical microscopy,[79] surface-enhanced Raman scattering [80] (including at the single-molecule level [81]), SPR microscopy, SPR spectroscopy,[82] *etc*. The optical properties of metal nanoparticles and their conjugates with biomacromolecules have been reviewed.[83–88]

The present review summarises data on different methods of synthesis and functionalisation of gold nanoparticles and their applications in biological studies. Particular attention is given to applications of colloidal gold particles in solid-phase assays, immunoassays and studies of biomolecules by vibrational spectroscopy (the use of gold nanoparticles in electron microscopic methods is only briefly mentioned). A special section is devoted to applications of gold nanoparticles as antigen carriers in immunisation.

II. Methods of preparation of colloidal gold

Methods of synthesis of CG (and other metal colloids) can be arbitrarily divided into the following two large groups: dispersion methods (metal dispersion) and condensation methods (reduction of the corresponding metal salts).

Dispersion methods for the preparation of CG are based on destruction of the crystal lattice of metallic gold in high-voltage electric field. If an electric arc is created in a liquid between two gold electrodes under electric field, its blazing leads to the mass transfer between electrodes accompanied by the colloidal gold formation. The yield and shape of gold particles formed under electric current depend not only on the voltage between electrodes and the current strength, but also on the presence of electrolytes in solution. The use of direct current leads to the formation of non-uniform gold particles. The addition of even very small amounts of alkalis or chlorides and the use of high-frequency alternating current for dispersion substantially improve the quality of gold hydrosols.

Condensation methods are more commonly employed than dispersion methods. Colloidal gold is most often prepared by reduction of gold halides (for example, of $HAuCl_4$) with the use of chemical reducing agents and/or irradiation (ultrasonic and UV irradiation, pulse or laser radiolysis).[47,89] Various organic and inorganic compounds serve as chemical reducing agents.[23] In the pioneering studies by Faraday and Zsigmondy,[3,4] formaldehyde, ethanol and white phosphorus were used, *inter alia*, as reducing agents. These compounds are still successfully used for the preparation of sols with an average particle diameter of 5–12 nm.[90,91] In addition, sodium citrate [92] (for the preparation of CG with an average particle diameter of 20 nm), ascorbic acid [93] (12 nm), ethylenediaminetetraacetic acid (EDTA) [8] (20 nm), sodium citrate in the presence of tannin (~5 nm),[94,95] sodium borohydride,[96] borohydride in a mixture with sodium citrate [97] or EDTA [98] and cyanoborohydride [99] (for the preparation of CG with an average particle diameter of ~5 nm) have found application in the synthesis of gold sols. Ultradispersed sols (the particle diameter of 2–3 nm) can be prepared with the use of sodium or potassium thiocyanate.[100] Other reducing agents, such as oxalic and tartaric acids, carbon monoxide, tin chloride, hydrazine, hydrogen peroxide, carbohydrates, phenols, aromatic aldehydes, essential oils and many other compounds (up to an aqueous extract of Dutch cigars),[90] which were extensively applied in the synthesis of CG early in the 20th century, are not presently used.

Zsigmondy and Svedberg [5,6] studied the kinetics of reduction of gold halide solutions and formulated the principal notions of the mechanism of formation of gold particles using reduction of $HAuCl_4$ with hydrogen peroxide as an example

$$2\,HAuCl_4 + 3\,H_2O_2 + 8\,KOH \longrightarrow 2\,Au + 3\,O_2 + 8\,KCl + 8\,H_2O.$$

This reaction produces only one electrolyte and, consequently, it can be monitored by conductometry.

According to Zsigmondy and Svedberg, ~30% of $HAuCl_4$ is initially reduced (to form a highly supersaturated gold solution in which the concentration of atomic metal is 6 orders of magnitude higher than that in a saturated solution), after which the reaction rate sharply decreases. The slow step involves condensation of gold to form very small particles (new phase nuclei). The latter form large but unstable aggregates (coagulation followed by peptisation), the sol solution turning blue. Particles involved in aggregates gradually enlarge and become centres of further rapid reduction of $HAuCl_4$. Once nuclei reach a particular critical size, a stable red sol is formed.

Taking into account the condensation mechanism of formation of a colloidal phase, several parameters determining the degree of dispersity of a gold suspension can be mentioned. Evidently, the rate of new phase nucleation depends on the concentrations of the reactants and the chemical nature of the reducing agent. At a low rate of nucleation and a rather high rate of particle condensation (the low degree of supersaturation of an $HAuCl_4$ solution), rather bulky particles are formed in small amount. At a high rate of nucleation and a rather low rate of particle condensation (the high degree of supersaturation of an $HAuCl_4$ solution), the probability of the formation of small particles increases. The increase in the concentration of the reducing agent is limited by aggregation stability of colloidal gold (rather high stability is achievable only in low-ionic-strength media).

In the case of self-nucleation, the larger the number of nucleation centres the higher the degree of dispersity of sols. In most cases, the nucleation and nucleus growth occur simultaneously (*i.e*., reduced metal is consumed simultaneously in both processes). A decrease in the nucleation rate also leads to the formation of more coarse-dispersed hydrosols; a decrease in the nucleus growth rate, to the formation of finely dispersed hydrosols. Studies of numerous gold sols prepared by chemical reduction demonstrated that most of them were characterised by low stability and a broad particle size distribution.[101] Relatively monodispersed particles with a diameter of 10–60 nm can be prepared only by the Frens method [40] using sodium citrate for reduction of $HAuCl_4$.

According to Zsigmondy, a sol with a narrow Au particle size distribution can be prepared by adding presynthesised Au seeds, on which condensation will occur, to an $HAuCl_4$ solution. In this method, sodium citrate or hydroxylamine can serve as reducing agents.[102–104] Isodisperse and isomorphous sols can be prepared only if the formation of new nuclei is prevented. As a rule, it is achieved by performing the process in two steps. Initially, a new phase nucleates, and then weak supersaturation is created in the sol, due to which new nuclei are not produced any more and only the already formed nuclei grow.[105]

Another procedure for the preparation of monodispersed gold sols was developed.[106] This method is based on reduction of a concentrated $HAuCl_4$ solution (0.5 mol litre^{-1}) by isoascorbic acid in the presence of gum arabic as the protective colloid. Under these conditions, condensation is accompanied by aggregation to form virtually monodispersed spherical gold particles with a diameter from 80 nm to 5 μm (depending on the acidity of the medium and the concentration of the reducing agent).

Gold nanoparticles can also be prepared by the two-phase microemulsion method. In the first step, metal-containing reagents are transferred from an aqueous to an organic phase. After the addition of a surfactant solution to this system, a microemulsion, *i.e*., a dispersion of two immiscible liquids, is formed. The reduction reaction proceeds in a dispersed phase in which the drop size is at most 100 nm. As a result, virtually monodispersed sols are formed.[107] Later, a single-phase method without the use of aqueous solutions was developed.[108]

In microemulsion methods of synthesis of CG, alkanethiols are often added to the reaction solution, and these additives form dense self-assembled monolayers on the gold surface. This method was employed for the preparation of self-assembled two- and three-dimensional ensembles of gold nanoparticles.[109–111] Some ligands, such as alkanethiols, amines, silanes, phosphines and halides, can be involved in digestive ripening, *i.e.*, in a process in which a polydispersed colloidal suspension is transformed into monodispersed state upon refluxing in a solvent containing surfactant ligands.[112]

In recent years, synthetic polymers, such as polyethylene glycol (PEG), polyethyleneimine, polyvinylpyrrolidone, poly(vinyl acetate), polyamidoamine (dendrimer), polydithiafulvene, chitosan, *etc.*, have found application in the synthesis of monodispersed CG.[113–117] Particles formed in the presence of these polymers are characterised by a higher size and shape uniformity.[118,119] Sodium borohydride,[120,121] alcohols and ethers,[122] hydrazine[123] and sodium diphenylaminosulfonate,[124] as well as ultrasonic radiation,[125] can be used for reduction. A tyrosine residue of a polyfunctional peptide served as a reducing agent.[126] Colloidal gold with the required particle size can be prepared by choosing a particular reducing agent and taking into account the characteristic features of the kinetics of formation of a colloidal phase.[127–130] A procedure for the synthesis of a marker for lectins with the use of PEG modified by carbohydrates and thiol groups was described.[131]

Physical (based on ultrasonic, UV, IR or ionising radiation or laser photolysis)[8,9,132–138] and electrochemical[139] methods of reduction are much less commonly employed than chemical methods. The advantages of the former methods are that impurities of chemical compounds are absent in the resulting sols (on the metal particle surface).[140]

In electron microscopic biological studies, gold clusters consisting of 11–67 atoms (undecagold, Nanogold ®) with a diameter of 0.82–1.4 nm are used as markers. These clusters are prepared by reduction of a gold triarylphosphine complex with sodium borohydride[11,141,142] or by reduction of $HAuCl_4$ in methanol with glutathione.[143,144]

The application of microorganisms and plant and animal cells for the CG synthesis is a new line of investigations in nanobiotechnology.[145–148]

In recent years, CG particles uniformly distributed in polymeric matrices and particles consisting of the metal core and a dielectric shell,[149–151] as well as non-spherical particles (spheroids, rods, chains)‡ and shell and planar structures,[152–162] have been much in demand due to the use of CG in biosensors. The latter structures can be prepared by reduction of gold salts in reverse micelles,[163] by the photochemical method[164] and with the use of porous aluminium or quartz templates and carbon nanotubes.[165,166] These methods were also employed for the synthesis of composite materials, for example, of gold–iron (possessing magnetic properties),[167] gold–silver[168,169] and other bimetallic nanoparticles.[47]

III. Functionalisation of gold nanoparticles

A gold hydrosol is a typical lyophobic colloid the particles of which bear a large negative surface charge (the surface potential is ~50 mV)[170] and, hence, it is stable only in very low-ionic-strength solutions. In lyophobic systems, the dispersion medium and the dispersed phase are substantially different in the chemical composition and the interface structure, as a result of which the surface forces at the interface are uncompensated. These systems are thermodynamically unstable and require special stabilisation.[17,170,171]

‡ It is known that the particle shape has a profound effect on their optical properties.

The stability of a sol can be increased by coating particles with a polymeric layer (conjugation with a polymer).[18–21,172] This method of stabilisation was proposed by Faraday, who studied the protective properties of gelatin.[3] The addition of even a small amount of a polymer (particularly, of polyelectrolytes) to lyophobic colloids can substantially increase their aggregation stability.

The formation of an adsorbed polymeric layer on the particle surface leads to a decrease in the interphase tension due to a strengthening of interactions between the dispersed phase and the dispersion medium, resulting in a substantial increase in the entropy component of the system (due to the involvement of molecules and ions of the surface layer in thermal motion together with particles of the dispersed phase). As a result, the aggregation stability of the system increases. The adsorption layer favours the formation of a solvate layer with the result that the particle surface becomes lyophilic and the lyophobic sol becomes much less sensitive to coagulation by electrolytes (due to electrostatic and hydrophobic interactions and structural mechanical stability).

The efficiency of stabilisation depends on solubility of the protective polymer in a dispersion medium, the ability of lyophobic particles to adsorb the polymer on their surface and the degree of the surface coverage by the polymer.

Not only the molecular mass of a polymer, but also the charge of its functional groups are of importance for the protective action. Polymers containing simultaneously acidic and basic groups (for example, protein analogues) or weakly basic polycationic macromolecules having the pH-dependent protective action are the most efficient stabilisers of gold hydrosols.[19,20,173]

The ability of polymers (including biospecific macromolecules) to stabilise lyophobic colloids underlies the preparation of stable bioconjugates, *e.g.*, complexes of CG with immunoglobulins, lectins, enzymes, hormones, lipoproteins, *etc.*[174]

The attachment of biospecific probes to CG can be performed by adsorption and chemisorption methods. Each method has characteristic features of its own. It is commonly accepted that stabilisation of CG by recognising biomolecules (functionalisation) occurs through passive adsorption of a polymer on the particle surface by electrostatic and hydrophobic interactions.[43,90] A strong negative charge of the gold particle surface provides their strong adsorption interactions with high-molecular-mass compounds. The presence of Coulomb interactions between the NH_2 groups of lysine residues of a protein adsorbed on the gold nanoparticle surface with the citrate ions was reported.[175]

In recent years, an important role of cysteine SH groups in binding of proteins to the surface of gold particles has been documented.[176]

The advantage of physical adsorption is that gold particles have a minimum effect on the structure of a macromolecule (a probe). Electrostatic (rather than covalent) interaction of a label with a probe ensures to the greatest extent the conservation of the native character of the probe and, consequently, the activity and specificity of its interactions with the target molecule. However, when using this procedure for the preparation of bioconjugates, the possibility of desorption of recognising molecules from the gold nanoparticle surface and their binding to the target molecules must be taken into account. The main distinguishing feature of polymer adsorption is that polymers are usually adsorbed in amounts substantially larger than those required for the formation of a monomolecular layer. Adsorption isotherms of polymer adsorption from dilute solutions show no inflection points, which could be indicative of the formation of a discrete monolayer, as is observed for low-molecular-mass surfactants. This is due to formation of a relatively large number of contacts between macromolecules and the surface. The simultaneous cleavage of these contacts is statistically improbable,[20] and consequently, in most cases, adsorption of individual macromolecules is irreversible.

Sulfur and gold atoms are known to form dative bonds.[22,177] Hence, it was proposed that alkanethiol linkers $HS(CH_2)_nR$ (R = COOH, OH or SO_3H; $n = 11-22$) were used to achieve stronger attachment of biomolecules to gold particles (the chemisorption method).[178] Interactions of these linkers with gold afford thiolates $[Au^0]_m \cdot Au^+S^-(CH_2)_nR$,[69] which form a monolayer on the particle surface.

In 1996, Mirkin *et al.*[73] performed thiolation of oligonucleotides at the 5′-end before their attachment to gold particles with a size of 15 nm. As a result, very stable conjugates (resistant to high and low temperatures) were prepared, which were employed for colourimetric determination of DNA in solution. Cyclic disulfides[179] and tris(ω-mercaptohexyl) derivatives[180] were used as linkers for the preparation of bioconjugates with gold particles with sizes of 30 and 100 nm, respectively. Conjugates with gold nanoparticles (nanospheres and nanorods) were prepared from thiolated proteins, immunoglobulins and avidin.[168,181] In addition to alkanethiols, ligands containing phosphino, amino or carboxy groups served as linkers.[47] The advantages of chemisorption are most pronounced in the case of linear molecules (for example, DNA). Adsorption of these molecules leads to the formation of structures characterised by close spatial orientation of the attached molecules.

However, non-covalent (adsorption) conjugation is still most commonly employed for the preparation of gold markers for immunochemical studies. In this approach, the native structure of biomacromolecules and, as a consequence, their functional properties are retained to the greatest extent.

IV. Colloidal gold in solid-phase assays

In early steps of development of immunoassay methods, preference was given to liquid-phase procedures. However, in recent years, solid-phase methods have found increasing use because they enable substantial simplification of experiments and a decrease in the background signal. For the first time, the solid-phase procedure was applied in radioimmunoassays of proteins.[182]

The problem is that the liquid-phase assays require the use of an antigen in solution; however, not all antigens are water-soluble.§ There is a large number of antigens, which can be transformed into the soluble state only in the presence of dissociating additives, such as sodium dodecyl sulfate, urea, guanidinium chloride, *etc*. These dissociating additives prevent the formation of immune complexes.[183] These difficulties can be avoided by immobilising antigens on solid supports. Microtitration plates and nitrocellulose (NC) filters have gained wide acceptance in solid-phase assays.[184]

Radioactive isotopes (^{125}I, ^{14}C and ^{3}H), enzymes (peroxidase, alkaline phosphatase, *etc.*)[185–187] and, in recent years, CG have found wide application as labels in membrane tests. In 1984, the use of CG as labels for solid-phase immunoassays was documented in several publications.[188–191] Colloidal gold conjugates have found application in solid-phase assays due to the intense red colour of gold-containing markers. The appearance of the colour in the course of the reaction enables visualisation of the process.[192–194]

Non-specific staining of proteins on NC filters was carried out using the gold stain AuroDye[195,196] (see¶). This method was further improved and modified.[197–199] Non-specific staining of proteins by gold stains is rather simple, convenient and highly sensitive.[200] The staining intensity is proportional to the logarithm of the protein concentration and its molecular mass.[201] Elution of CG-immobilised proteins and the photometric determination of the protein concentration from the light absorption of the resulting complex at 540 nm were described.[202] The gold stain was used also for direct protein staining in gels,[203] for detection of nucleic acids immobilised on blots,[204] for protein determination by electron microscopy[205,206] and for the colourimetric determination of proteins in solution.[207]

In assays, an antigen immobilised on a membrane is incubated with a solution of the corresponding CG-labelled antibodies (or other biospecific probes). Immunoglobulins,[189,191,208,209] Fab and scFv fragments of antibodies,[123,210] protein A,[188,190] lectins,[211,212] enzymes,[204] streptavidin or antibiotin antibodies (in studies of biotinylated specimens),[213,214] *etc*., were applied as probes in 'gold' dot-blot assays. To visualise different antigens on a membrane, several labels can simultaneously be used (for example, CG and peroxidase[215,216] or alkaline phosphatase[217]). Colloidal gold conjugates also have found application in tissue blots,[218] in which tissue prints are blotted onto nitrocellulose filters and the sought-for components are visualised with labelled probes, as well as in linear blots.[219]

In membrane tests, CG was used for diagnosis of parasitic,[220–223] viral[224–227] and fungal[228,229] diseases, tuberculosis,[230] melioidosis,[231] syphilis,[232] brucellosis[233] and shigellosis,[234] for the determination of early pregnancy[235] and blood groups,[236] for the identification of plant antigens[237,238] and for dot-blot hybridisation.[239]

Attempts were made (in our opinion, not quite convincing) to stain samples with CG in wells of microtitration plates[240–244] or on the cover glass surface[245] and to use colloidal silver[246] or coal particles[247] instead of CG in solid-phase immunoassays. The application of CG in assays of large series of antigens in micromatrices (immunochips) holds more promise.[248] The latter method enables the determination of analytes at a concentration of 60–70 ng litre^{-1}. In this case, up to 384 samples can simultaneously be analysed using microlitre amounts of an analyte and an immunogold marker as the detecting agent.

Depending on the form in which an antigen is immobilised on a membrane, immunodot and immunoblot assays are distinguished. The former is employed in assays of non-fractionated specimens; the latter, in assays of pre-fractionated specimens. The immunodot assay is one of the simplest methods of analysis of antigens immobilised on membranes and, in some cases, enables their quantitative estimation. The immunodot assay is most often used for soluble antigens.[249] Studies of whole bacterial cells by dot assay with an enzymatic label or by colony blot assay were also documented.[250–253] The dot assay of whole bacterial cells using visualisation of the reaction products with biospecific markers, *viz*., CG conjugates, (cell-gold immunoblotting) was used[254,255] for serotyping of nitrogen-fixing soil microorganisms. More recently, this method was applied to the diagnosis of intestinal infections.[256]

The sensitivity of a label depends on the gold particle size [the light attenuation coefficient increases with increasing particle diameter (> 40 nm)].[257] This is the reason why most of companies producing gold markers recommend to use conjugates of a probe with CG particles with a diameter of 20–30 nm in blot assays. The sensitivity in dot assays was increased by using conjugates of recognising molecules with gold nanoparticles as nanorods and nanoshells.[258,259]

In the 1990s, some companies began to produce immunochemical diagnostic test kits. Due to high specificity and sensitivity of the immunoassay technique, these tests have found wide application in the determination of narcotic drugs and toxins, the early pregnancy diagnosis and screening of especially dangerous infections and urogenital diseases.[260–267] In recent years, procedures have been developed for DNA hybridisation[268] and tuberculosis diagnosis.[269] Immunochromatography is a technique for rapid immune tests, which is most actively developed and widely used in practice.

Immunochromatographic assays are based on the motion of an eluent along a membrane (lateral diffusion), which is accom-

§ For example, a wide class of corpuscular antigens, such as whole bacterial cells, are among water-insoluble antigens.

¶ The gold stain is a gold sol partially stabilised by polymers, such as PEG 20M, Tween-20, *etc*.

panied by the formation of specific immune complexes in different membrane regions visualised as coloured bands.[270] Enzymes, coloured latexes and, most often, CG are used as labels in these systems.[271, 272]

Examinations of these test systems demonstrated their high stability, reproducibility of the results and correlation with alternative methods. The degree of inhomogeneity of the detected bands estimated by densitometry is in the range of 5%–8%, which enables reliable visualisation of the results of assays. These tests are very simple and convenient in use. However, the technology of production of components of such test kits (unlike dot-blot tests) is rather complicated and is the property of manufacturers, which limits the use of these methods in routine laboratory practice.

V. Application of colloidal gold for the quantitative protein determination

Dot-blot assays with the use of CG conjugates have found increasing use and gained wide acceptance due to high sensitivity and simplicity. However, this method has some limitations, among which are difficulties of rigorous quantitative interpretation of the results and operations with low-molecular-mass ligands and considerable problems associated with the detection of components of complex biological systems (homogenates, exudates, *etc.*).

In 1980, Leuvering *et al.*[273] developed a new immunoassay method called the sol particle immunoassay (SPIA). This method employs two important properties of gold sols: (1) the typical bright red colour of sols, which remains virtually unchanged upon adsorption of high-molecular-mass compounds on gold particles, and (2) changes in the colour of sols upon aggregation of gold particles, which is detected from changes in absorption in the visible spectral region. These changes in absorption are clearly detected both spectrophotometrically and visually.

The SPIA method is rather simple in use. Solutions of a gold-containing marker and the sample under study are mixed in a reaction vessel, and either the spectrophotometric characteristics of the suspension are monitored or the colour of the suspension is visually estimated during the incubation period (0.5–2 h). If the biospecific reaction proceeding on the CG surface is accompanied by destabilisation of the sol resulting in aggregation of gold particles (in the case of polyclonal antibodies, this process is called agglutination), the absorption spectrum of the suspension shows substantial changes associated with a noticeable change in its colour (from red to blue or gray). Antigens can be quantitatively determined with spectrophotometers and colourimeters.

Later,[274–277] this method was modified and optimised to detect human chorionic gonadotropin in the urine in pregnancy (larger gold particles and monoclonal antibodies to different antigen regions were used). Based on the results of these investigations, home-use test kit Discretest ™ for early pregnancy is produced (Chefaro company, the Netherlands). The immunocolourimetric test kits for the rheumatoid factor and streptolysin is produces by PLIVA Lachema company (Czech Republic).

More recently, this method was applied to immunoassays of *Schistosoma*[278] and *Rubella*[279] antigens, the determination of affinity constants for different isotypes of mouse monoclonal IgG[280] and quantitative determination of immunoglobulins[281, 282] and cystatin C (an endogenous marker for the filtration ability of kidney glomeruli).[283]

In 2002, Thanh and Rosenzweig[284] rediscovered this method and applied it to the quantitative determination of antibodies to protein A with the use of the conjugate of protein A with CG. In recent years, new methods for the detection of a response of a system (for example, antigen–antibody labelled by CG) to interactions, such as photothermal spectroscopy,[285] double-beam laser absorption spectroscopy[286] and hyper-Rayleigh scattering,[287] have enabled an increase in the assay sensitivity. The combined use of agglutination of latex and gold particles (heterogeneous SPIA) was proposed.[288]

All versions of the SPIA method are very simple in use and are characterised by high sensitivity and specificity. However, researchers faced with the fact that antigen–antibody interactions on sol particles not always lead to destabilisation of the system (particle aggregation). In some cases, in spite of the obvious pair complementarity, the colour of the solution and, consequently, the absorption spectra change only slightly, if at all.

After a series of preliminary experiments, it was hypothesised[289] that in these cases a second protein layer is formed on gold particles without loss of aggregation stability of the sol. In this case, changes in the spectra due to adsorption of the second biopolymer and, correspondingly, changes in the structure of the biopolymer layer on the metal particle surface are relatively small. However, even such small changes in the absorption spectra can be recorded and used for quantitative assays in biological applications.[290, 291]

Another version of the above-described method was proposed by Mirkin and co-workers.[73] This version is based on the colourimetric determination of polynucleotides interacting with complementary oligonucleotides immobilised on gold nanoparticles. This process is accompanied by the formation of an ordered three-dimensional structure of gold nanoparticles, which leads to changes in the absorption spectrum of the solution and is detected visually[73] or photometrically.[292]

The formation of ordered three-dimensional structures in the reactions of IgG-functionalised gold nanoparticles with antigens[176, 293] or with aptamers was documented.[294] It was demonstrated that biologically programmed ensembles of gold nanoparticles can be prepared with the use of an avidin–biotin system.[295, 296] An analogous approach was applied for the detection of lectins.[146] Ca^{2+}-dependent aggregation of gold nanoparticles coated with carbohydrates was described.[297]

In spite of the fact that the SPIA method is very simple in use, the real practical applications of this method during 20 years have been described in rather modest number of papers. Only in the very recent past, this method has been demonstrated to be promising as a high-performance clinical test. In our opinion, the SPIA method has little use because the physicochemical and optical mechanisms of the conversion of signals, which are generated upon the biospecific binding of target molecules to CG conjugates, into characteristics of light attenuation or scattering by suspensions are poorly understood.

A new version of the SPIA method with the use of microtitration plates and microplate reader and a trypsin (proteolytic enzyme) conjugate with CG as a specific visualising agent for proteins was proposed.[298] This assay holds considerable promise for the rapid, sensitive and quantitative protein determination.

VI. Application of gold nanoparticles in studies of biologically active compounds by vibrational spectroscopy

Raman spectroscopy is an efficient tool to investigate molecular structures because the set of intramolecular vibrational frequencies is uniquely related to the molecular structure as well as to intermolecular and intramolecular interactions.[299, 300] However, since the intensity of the Raman signal is relatively low, the detection of this signal is a rather complex problem and requires the use of modern laser sources and photon counting systems. In 1974, Fleischman *et al.*[301] suggested that the effective number of molecules involved in scattering from the adsorbed monolayer could be increased by increasing the real surface area with retention of the 'visible' surface area illuminated with pumping radiation. For this purpose, the silver surface was made coarse by anodic etching and the spectra of compounds adsorbed on this surface were recorded. The intensity of Raman scattering increased by a factor of 10^6–10^7, while the surface area after

etching of the silver electrode surface increased by only an order of magnitude.[301] More recently, it was demonstrated that the observed enhancement of Raman scattering is associated with a new non-linear effect called the surface-enhanced Raman scattering (SERS) effect.†

Surface-enhanced Raman scattering has some features distinguishing it from conventional Raman scattering. First, SERS cross-sections for vibrational modes of adsorbed molecules can be increased by a factor of 10^{10} or more compared to the analogous parameters for unadsorbed molecules. Second, the enhancement of scattering varies depending of the excitation frequency and the degree of roughness of the support according to a law specific for SERS. Third, the SERS spectra of many adsorbed molecules are substantially different from the corresponding Raman spectra of molecules in the free state. This is manifested in the selective enhancement of particular vibrations and the appearance of new bands in the SERS spectrum.

Abundant information on applications of surface-enhanced Fourier-transform IR and SERS spectroscopy in biological studies is summarised in the reviews.[303–306] Vibrational spectroscopic methods are used for studying individual biomolecules, as well as cells and tissues.[307–311]

In most of the cited studies, vibrations of biomolecules were enhanced due to their adsorption on metal electrodes or thin films. However, in recent years the methods in which signals are enhanced with the use of colloidal metals (primarily, gold and silver), have gained wide acceptance.[312–316] In these methods, the signal is enhanced by giant non-linear local fields that are produced in metal clusters.[317,318] Different versions of immunoassay with the use of antibodies or antigens adsorbed on gold nanoparticles and vibrational spectroscopic methods were developed.[319–323]

It was demonstrated for the first time [324] that Fourier-transform IR spectroscopy can be employed for the sensitive determination of interactions of protein molecules with the gold particle surface and for the reliable and simple monitoring of biospecific reactions. This method (that is called spectroimmunochemical) can serve as a basis for the development of test systems detecting biospecific antigen–antibody, enzyme–substrate, lectin–polysaccharide, *etc.* interactions. The observed changes in the spectra of biomolecules immobilised on gold particles are evidence that these biomolecules are indeed adsorbed. The proposed method along with other methods (for example, SERS) can be used for controlling the quality of gold conjugates.

VII. Gold nanoparticles as antigen carriers

Let us briefly consider two interrelated problems of modern immunology that attract the attention of many researchers. These are the preparation of antibodies against non-immunogenic low-molecular-mass compounds (haptens) and the design of new generation vaccines based on natural (microbial) or synthetic peptides.[325–328]

The biosynthesis of antibodies in organisms is known to be induced by compounds possessing a rather developed structure (immunogenicity). These compounds include proteins and polysaccharides.[329] However, many biologically active compounds (neuromediators, hormones, vitamins, antibiotics, *etc.*) have rather low molecular masses. Low-molecular-mass antigens belong to the so-called weak antigens, *i.e.*, a pronounced immune response against these antigens (biosynthesis of the corresponding antibodies) is not developed.

The preparation of antibodies against haptens has attracted interest for several reasons. First, antibodies to particular fragments of biomacromolecules can serve as a highly efficient tool for investigating their topography and structures. Second, the preparation of antibodies to such low-molecular-mass compounds, as antibiotics, hormones and some pharmaceuticals, would enable the control of their amount in the blood of patients, in meat and dairy products and in cultural media. The drug monitoring is widely used in all developed countries. This method enables substantial enhancement of the efficiency of curing and prevention of complications. Among various procedures for the detection of such compounds, immunochemical assay is most convenient and highly sensitive, which implies the preparation of antibodies to these low-molecular-mass compounds. Antibodies to low-molecular-mass compounds hold considerable promise in immunotherapeutic practice.[325,330] Third, attempts are made to use synthetic peptides for the design of artificial (acellular) vaccines containing only protective antigens and inert carriers.

Since haptens exhibit weak immunogenicity, the choice of an optimal carrier (a delivery system) providing a high immune response and the preparation of sufficiently pure antibodies are important problems to be solved in the preparation of antibodies to haptens. These problems are traditionally solved by covalent coupling of a hapten to a protein matrix, the so-called schlepper (which comes from the German word 'schleppen', which means 'to drag, lug') with the use of adjuvants and intensive schemes of animal immunisation by the resulting conjugate.[325,331] Bovine serum albumin, ovalbumin, thyroglobulin, hemocyanin, diphtheria or tetanus anatoxins (for synthetic peptides), *etc.* are usually used as schleppers. However, these processes give antibodies against both a hapten and immunodeterminant sites of a carrier. The use of such carriers not necessarily gives rise to a pronounced immune response to weak antigens. In addition, the subsequent purification and screening of the resulting antibodies are time-consuming and expensive, and their titer and affinity are often low.

Most of the presently used adjuvants based on oil emulsions and suspensions of inorganic compounds are, as a rule, prone to phase separation, their immunogenic properties change with time, and many of such adjuvants cause local and systemic toxic effects.[332,333] The application of antibody phage display is seemingly the most promising solution of the problem of the preparation of antibodies against haptens.[334] However, this technique is virtually inapplicable in vaccinology.

In recent years, studies have been carried out on the design of the so-called complex antigens, *i.e.*, artificial macromolecular complexes containing both the desired antigenic determinants and carriers and/or adjuvants. In particular, synthetic polyelectrolytes [poly-L-lysine, polyacrylic acid, polyvinylpyridine, poly(styrenesulfonate), Ficoll, *etc.*] were proposed as carriers.[335] For example, the influenza vaccine Grippol was designed based on the cation exchange resin 'Polyoxidonium'. These polymeric compounds are synthesised by radical polymerisation of the corresponding monomers.[336] The simplicity of the chemical synthesis of polyelectrolytes and the possibility of preparation of polymer chains in a broad molecular mass range (*i.e.*, of different length), solubility in water and other properties (the ability to undergo conformational transformations, complex formation with proteins, *etc.*) opened up possibilities for their application in immunological studies.[337] These carrier-adjuvants can form a depot of antigens at the site of injection, enhance the presentation of antigens to immunocompetent cells and induce the production of required cytokines. However, low immunogenicity of such complexes associated with a low epitopic density stimulated a search for new non-toxic and efficient carriers having, in addition, adjuvant properties.

In our opinion, nanosized corpuscular carriers, such as polymeric nanoparticles [for example, poly(methyl methacrylate)],[338] liposomes, proteosomes and microcapsules,[339–341] fullerenes,[342,343] carbon nanotubes,[344] *etc.*, hold considerable promise in this respect. The use of such carriers leads to a change in the form of manifestation of immunogenicity of the required compound in the immune system of host organisms. An antigen

† Adsorption of molecules of the compound under study on the metal surface is accompanied also by changes in luminescence, absorption and non-linear effects and the appearance of induced optical activity.[302]

adsorbed to, or encapsulated in, nanoparticles can be used as an adjuvant for optimisation of the immune response of the organism upon vaccination. For example, an analysis of the dynamics of an immune response demonstrated that the titer of antibody in response to an antigen introduced together with fullerenes was equal to the titer of antibody in response to the antigen introduced together with the Freund's complete adjuvant (FCA) and was approximately an order of magnitude larger than the titer of antibody in response to the antigen introduced without an adjuvant.

In 1986, the successful preparation of antibody to glutamic acid with the use of CG particles as a carrier was reported in the pioneering study by Japanese researchers.[345] (It should be noted that colloidal metals were used for stimulation of an immune response to haptens and complete antigens as early as 1920th by Zil'ber and co-workers.[346–348]) More recently, this method has been applied and improved for the preparation of antibodies to the following haptens and complete antigens: amino acids,[349, 350] platelet activating factor,[351, 352] quinolinic acid,[353] biotin,[354] immunophilin,[355] lysophosphatidic acid,[356] endostatin,[357] peptides of hepatitis C virus capsid,[358] surface antigens of *Yersinia*[359] and brucelles.[360] In all the above-mentioned studies, haptens were directly conjugated with CG particles, mixed with FCA and used for immunisation of animals. As a result, high-titer antisera, which did not require further purification from ballast antibodies, were obtained.

In 1993, Australian scientists[361] suggested that haptens should be added to a carrier protein before conjugation with CG. More recently, this approach was applied for the preparation of antibodies against a number of synthetic[362–365] and natural[366] peptides, amino acids,[367, 368] citrulline[369] and phenyl β-D-thioglucuronide.[370] In these studies, FCA or *N*-acetylmuramyl-L-alanyl-D-isoglutamine were used as adjuvants.[363] Antibodies prepared according to this procedure have high specificity for antigens under examination and a higher titer (the authors of the publication[361] referred to this titer as extremely high), from 1 : 250 000 to 1 : 1 000 000, compared to antibodies prepared according to a conventional procedure (by conjugation with a schlepper).

In 1996, researchers from the Khabarovsk Research Institute of Epidemiology and Microbiology demonstrated[371] for the first time that CG particles in a viral vaccine can be used as carriers of tick-borne encephalitis virus capsid protein antigen. According to the results of this study, in spite of the fact that no adjuvants are present in the vaccine, the latter has higher protective properties compared to commercial analogues. In 2003, a new procedure for the preparation of antibodies against Aβ peptides, which is a molecular marker of Alzheimer's disease, was described.[372] The authors designed a molecular mimic of the fibrillar form of Aβ peptides by covalently binding prethiolated oligomers to the gold particle surface. Antibodies to antigens that were synthesised according to this procedure, have very high specificity and affinity for both soluble and fibrillar forms of Aβ peptides.

Numerous studies were devoted to applications of CG particles in the design of DNA vaccines. In DNA immunisation, gene constructions coding proteins to which it is necessary to prepare antibodies, are introduced into organisms. In the case of efficient gene expression, these proteins serve as antigens for the development of an immune response.[373, 374] In early studies, immunisation was performed with subcutaneous or intramuscular injections of 'naked' DNA. However, the biolistic transfection with nanoparticles began to be applied for this purpose almost at the same time. This procedure proved to be very efficient due apparently to multiple sites of interactions between a transgene and tissues. The injection material began to be administered subcutaneously, epicutaneously or intranasally.[375, 376]

Among nanoparticles, colloidal gold particles are most widely applied as DNA carriers.[377–383] Initially, gold has been used only as a carrier, but more recently gold was demonstrated to enhance an immune response *in vivo*.[384] However, to our knowledge, data on the mechanisms of this action of gold particles are lacking.

Gene immunisation (it is often called DNA immunisation), which has been developed in tests on animals, is highly efficient, particularly, against viral infections, such as tick-borne encephalitis, HIV infection, hepatitis B and some other. The DNA immunisation has some advantages over conventional vaccination. One recombinant vector can simultaneously direct the synthesis of several antigens, which decreases the number of immunisations. This eliminates problems associated with the difficulties of penetration of proteins into organisms and, in addition, substantially decreases the risk of side effects, which depend on toxicity of inactive proteins introduced in the case of conventional vaccination or virulence in bacteria and viruses. In the coming years, DNA immunisation would be expected to be among the most efficient methods of gene therapy.[37, 385, 386]

The preparation of antibodies to complete antigens and haptens of different nature using CG particles as carriers was documented.[387, 388] It was demonstrated that CG as an antigen carrier activates phagocytic activity of macrophages and influences the functioning of lymphocytes, which is apparently responsible for its immunomodulation effect. The most interesting aspect of manifestation of the immunogenic properties by haptens upon their immobilisation on colloidal gold is that CG particles act simultaneously as adjuvants and carriers, *i.e.*, present haptens to T-cells. Gold nanoparticles conjugated with antigens were found to influence activation of T-cells (an increase in proliferation by a factor of 10 compared to that upon the addition of the native antigen). This fact shows that the targeted activation of T-cells (for example, by antigens of *Mycobacterium tuberculosis*, HIV, *etc.*) followed by activation of macrophages and pathogen killing is possible, in principle, which holds considerable promise for the design of new generation vaccines.

The question is what is responsible for the adjuvant properties of CG. Nowadays, there is no answer to this question. However, let us give some comments.

In our opinion, conclusions about the preferable macrophage response to corpuscular antigens, unlike soluble antigens, is undoubtedly valid.[361] This fact was confirmed in the studies[379, 381, 384] where the mechanism of action of DNA vaccines was elucidated and gold particles were used for delivery of genetic material into cells. In these studies, the role of the Kupffer cells and the Langerhans islets in the immune response was revealed. Moreover, CG particles were used in the studies[389, 390] of endocytosis by macrophages. (Gold particles included in phagocyte lysosomes even received their own name 'aurosomes'.) The influence of dendrite cells on the immune response to antigens conjugated with gold nanoparticles was discussed.[391] In addition, the authors mentioned that, when using nanoparticles in medical practice, it is necessary that lipopolysaccharides be absent on their surface.

However, these data do not reveal further mechanisms of presentation of antigens to T helpers. According to modern views,[329, 392] the presentation of antigens to T-cells is preceded by processing (protein cleavage into peptide fragments) followed by the formation of compounds with molecules of the major histocompatibility complex, which performs the delivery of an antigen fragment to the surface of antigen-presenting cells. Hence, it remains unclear how this process can occur with haptens. The assumption about the existence of a multivalent antigen, *i.e.*, the antigen that is formed due to a high local concentration of monovalent antigens on the gold particle surface, also does not answer this question.

At the same time, the influence of gold salts on the immune system has long been known.[393–397] Treatment of a number of autoimmune diseases, in particular, of rheumatoid arthritis, is based on the ability of gold to change non-specific immune reactions of an organism. For example, the successful therapy of rheumatoid arthritis by a colloidal gold solution was documented.[30, 398] According to the results of another study,[399] in this

case the function of CG is to inhibit monocyte-induced proliferation of lymphocytes. The transformation of Au(0) into Au(I) in immune system cells under the action of certain amino acids was discussed.[400]

Contradictory results were obtained in the studies[401–403] aimed at examining the sensitivity of mammalian organisms to the introduction of metallic gold. In particular, it was noted[401,402] that the injection of CG into laboratory animals can lead to inflammatory reactions, accumulation of gold in reticular cells of lymphoid tissues and activation of cellular and humoral immunity. However, in the study[403] dealt with the influence of gold nanoparticles on the immune system cells, it was concluded that CG particles is a non-cytotoxic, non-immunogenic and biocompatible material for the potential application in different fields of nanoimmunology, nanomedicine and nanobiotechnology.

In recent years, the use of gold nanoparticles for targeted drug delivery has been considered in a number of publications (see, for example, Refs 32 and 404–406). In this connection, it should be noted that, in our opinion, this problem must be considered with caution taking into account that antibodies against the administered drug adsorbed on CG particles can be formed in animal and human organisms.

In conclusion, it should be noted that it is apparently the time to consider not only biochemistry, but also biophysics of the immune response, because it is the unique biophysical properties of metal particles, in particular, the surface charge and the electrostatic field of the particles (which influence the charge, orientation and polarisation of antigenic molecules adsorbed on the particles) that have a substantial effect on the immune response.

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