Advances in nanotechnology have enabled the development of multifunctional nanoparticles that can simultaneously perform various functions, including targeting, imaging, and therapy. Recently, gold nanoparticles (AuNPs) have been investigated for potential multifunctional uses in nanomedicine as therapeutic agents and drug carrier, although the mechanisms of interaction with drugs are still little known. In the present work, we studied the interaction between functionalized hydrophilic AuNPs and the immune-system suppressant drug Methotrexate (MTX) at molecular level. The aim was to define the overall structure of drug loaded AuNPs and drug location on the colloidal nanoparticles surface, that will improve drug efficacy and knowledge of the pharmacodynamics and pharmacokinetic properties. Small and monodisperse (=5±1 nm) AuNPs were synthetized by a wet chemical reduction method using hydrophilic thiol 3-mercapto-1-propanesulfonate (3MPS) as a functionalizing/capping agent. AuNPs-3MPS@MTX conjugate was obtained by post-synthesis procedure via noncovalent interaction. Optical, structural and morphological properties of the AuNPs-3MPS@MTX bioconjugate and the AuNPs alone were obtained through the use of conventional techniques (UV-Visible, FT-IR, DLS), supported by 1D- and 2DNMR studies. Gold colloids before and after interaction with MTX were further characterized using Atomic Force Microscopy (AFM), High Resolution Transmission Electron Microscopy (HR-TEM), Synchrotron Radiation-induced Xray Photoelectron Spectroscopy (SR-XPS) and Small-Angle X-ray Scattering (SAXS). The results suggested MTX was successfully loaded on the negatively charged nanoparticles surface via electrostatic bonds, leading to the formation of large clusters with close packed arrangement of AuNPs-3MPS@MTX. These observations are of importance for delivery purpose and can soon provide considerable contribution to nanomedicine.